Risk Assessment of Norovirus on Shellfish from Indonesian Fish Markets

By

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Declaration of Originality

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Statement of Co-Authorship

This thesis comprises of work that has been prepared to be submitted to journals. Information for each chapter is provided in the section of communications arising from this thesis.

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Author 1 contributed 70% (designed the experiment, optimized the improved method, conducted laboratory analysis, analysed the data and wrote the manuscript), author 2, 3 and 4 each contributed 10% (provided suggestion for the experimental design, contributed to the development of molecular method and provided input for the manuscript).

We the undersigned agree with the above stated "proportion of work undertaken" for the above prepared peer-reviewed manuscript contribute to this thesis.

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List of Abbreviations

ASP	Amnesic Shellfish Poison
BIOHAZ	Biological Hazard
Bps	Base pairs
BSN	Badan Standarisasi Nasional [National Standardization Agency of Indonesia]
$CaCl_2$	Calcium Chloride
CFU	Colony Forming Unit
ClO_2	Chlorine dioxide
Ct value	Cycle threshold value of real-time PCR
ddH_2O	Double-distilled water
DEPC	Diethyl Pyrocarbonate
DT	Digestive tissues
D value	Time required to a $\log_{10}$ reduction (min)
E. coli	*Escherichia coli*
EFSA	European Food Safety Authority
FAO	Food Agriculture Organization of United Nations
FAOSTAT	FAO Statistic database
FCV	Feline Calicivirus
GHP	Good Handling Practices
GMP	Good Manufacturing Practices
GII	Genogroups II
HAV	Hepatitis A Virus
HBGAs	Histo-Blood Group Antigens
HEV	Hepatitis E Virus
HPP	High Pressure Processing

ISC RT-qPCR	*In Situ* Capture Reverse Transcription Quantitative Polymerase Chain Reaction
Kb	Kilobases
LAMP	Loop-Mediated Isothermal Amplification
LB	Lactose Broth
LTFC	Long-Term Facilities Care
MIQE	Minimum Information for publication of Quantitative Real-Time PCR Experiments
MMAF	Ministry of Marine Affairs and Fisheries of Republic Indonesia
MNV	Murine Norovirus
MPN	Most Probable Number
MS2	MS2 bacteriophage
NoV	Norovirus
ORF	Open Reading Frames
P1	Protruding 1 domain of viral protein
P2	Protruding 2 domain of viral protein
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
PGM-MB	Porcine Gastric Mucin-conjugated Magnetic Beads
PMA	Propidium Monoazide
PMAxx™	Improved version of PMA by Biotium®
PSP	Paralytic Shellfish Poison
P (value)	Probability value or significance
PV	Poliovirus
QMFSRA	Quantitative Microbial Food Safety Risk Assessment
QMRA	Quantitative Microbial Risk Assessment
R^2	The coefficient of determination

RdRp	RNA-dependent RNA polymerase
RMSE	Root Mean Square Error
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNasin	Ribonuclease Inhibitor
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
S	Shell domain of viral protein
SaV	Sapovirus
SMV	Snow Mountain Virus
SNI	Standar Nasional Indonesia [Indonesian National Standard]
SSOP	Sanitation Standard Operational Procedure
TaqI	a restriction enzyme isolated from the bacterium *Thermus aquaticus*
TE	Tris EDTA
TIA	Tasmanian Institute of Agriculture
TuV	Tulane Virus
UV	Ultra Violet
VLPs	Virus-like particles
VP1	Viral Protein of major capsid
VP2	Viral Protein of minor capsid
VPg	Viral Protein genome-linked
WWF	Word Wide Fund for Nature
z value	Changes in temperature needed to produce 90% change in the reduction rate (D value)

Abstract

Norovirus (NoV) infection is estimated to cause almost 20% of acute gastroenteritis cases worldwide. Infants, the elderly and the immunocompromised are those most susceptible to NoV infection. NoV is known to be persistent in the environment for long periods (60-80 days at 25°C), is infectious at low doses (at 8 – 2,800 viral particles), can be shed at high concentration (up to 10^9-10^{11} viral copies per gram faeces of infected person), and is mainly transmitted through the faecal-oral route. Therefore, a small amount of NoV contamination in the environment, water or food can cause large outbreaks.

Shellfish, in particular, are susceptible to NoV contamination because they filter large amounts of water and accumulate different types of suspended particles including bacteria and viruses when grown or harvested from contaminated areas. In Indonesia, some shellfish growing and harvesting areas are located close to estuaries which can be contaminated by untreated domestic sewage effluent, especially during flood incidents. Even though shellfish in Indonesia are mostly consumed cooked, inadequate cooking and cross-contamination during food preparation steps can lead to NoV contamination in the prepared meal.

Risk assessment of NoV, especially in shellfish from Indonesian markets, remains challenging due to the lack of prevalence data, no recorded NoV outbreaks caused by shellfish consumption, and the lack of knowledge of the efficacy of post-processing steps including handling and cooking based on consumer behaviour in Indonesia. Boiling, stir-frying and steaming are the most common cooking practise of shellfish in Indonesia which can reduce the NoV contamination. In case the shellfish is being consumed as a raw or fresh product, the use of disinfectant such as Chlorine Dioxide (ClO_2) to reduce the viral contamination or to prevent cross-contamination during post harvesting or handling is a potential risk management strategy. In addition, standard quantification assays for NoV based on the cell-culture system are as yet unavailable. Therefore, NoV studies rely on molecular based methods such as Reverse Transcription Polymerase Chain Reaction (RT-PCR).

This project optimised a Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) method to obtain prevalence data on NoV in shellfish from Indonesian markets and further utilised a NoV surrogate (MS2 bacteriophage, 'MS2') for inactivation studies, to fill those data gaps. The results provided better understanding of NoV prevalence and survival and could be used to predict the risk of NoV contamination in shellfish from Indonesian markets.

The first aim of this thesis was to evaluate the application of RT-qPCR after pre-treatment with enzymes because current methods quantify both infectious and non-infectious viral particles and may over-estimate the risk of viral infections, especially in the inactivation and prevalence studies. Therefore, sample pre-treatments are required to differentiate the infectious from non-infectious viral RNA.

MS2, a cultivable NoV surrogate was used in this study. RT-qPCR after pre-treatment with RNase followed by RNasin showed better performance than RNase alone or TaqI in the elimination of the RNA from inactivated MS2 and produced a comparable result to the plaque assay. This modified RT-qPCR method was shown to be applicable for the quantification of infectious MS2 after inactivation treatment by heat or ClO_2, producing comparable results to plaque assays.

The next aim of this thesis was to compare the inactivation kinetics of NoV and MS2 treated by heating and ClO_2 in buffered media (PBS solution) and the shellfish matrix, as the NoV surrogates may have different inactivation kinetics compared to NoV. The efficacy of both inactivation methods was also determined.

To provide artificial contamination of NoV and MS2 in the mussel for heat treatment studies, bioaccumulation process of the viruses in Tasmanian Blue Mussel (*Mytilus galloprovincialis*) was done to mimic the actual virus transmission routes in shellfish. While for ClO_2 treatment studies, the mussels were artificially contaminated by dipping the tissue in solutions of NoV and MS2 for 30 min to represent the cross-contamination process. NoV and MS2 in buffered media and bioaccumulated mussel were heated at 60, 72 and 90° C at various times. The evaluation of NoV and MS2

inactivation kinetics showed that the Weibull model performed better in estimating the survival of NoV and MS2 in buffered media, while the Biphasic model provided better estimation of virus survival in mussel matrix. The D values of NoV were generally higher than MS2 in both buffered medium and mussel matrix, showing a higher resistance of NoV towards heat treatment. Furthermore, for all temperatures, inactivation of both viruses in mussel matrix required a longer time to achieve 1 $\log_{10}$ reduction compared to inactivation in buffered media.

The efficacy of chlorine dioxide (ClO_2) to inactivate NoV and MS2 in buffered media and artificially-contaminated mussel was studied using ClO_2 at 10, 20 and 40 ppm with various exposure times at 25°C. The result showed that 40 ppm ClO_2 treatment reduced NoV and MS2 more significantly in both buffered media and mussel matrix than 10 and 20 ppm treatments. In general, the virus reduction was higher in buffered media than in mussel matrix for all ClO_2 treatments. For example, the reduction of MS2 in buffered media treated with 40 ppm ClO_2 for 200 min resulted in > 6 $\log_{10}$ PFU/ml reduction, while in mussel matrix the same treatment only reduced MS2 by < 3 $\log_{10}$ PFU/g. At the same treatment, NoV in buffered media were reduced for more than 3 $\log_{10}$ copies/ml, while only 2.36 $\log_{10}$ copies/g reduction was observed in mussel matrix. The inactivation of ClO_2 of both viruses in buffered media and mussel matrix was equally well described using the quasi-mechanistic Hom model or the Weibull model.

The first prevalence data for NoV GII in shellfish in Indonesia are presented in this thesis. The data are for three shellfish species *i.e.* Green Mussel (*Perna viridis*), Blood Cockle (*Anadara granosa*) and Oriental Hard Clam (*Meretrix lusoria*), that are commonly consumed in Indonesia. Shellfish were sampled from four fish markets in Jakarta and Panimbang, Indonesia, in July 2016 and 2017. The NoV from extracted digestive tissue (DT) of shellfish was enumerated using the enzymatic pre-treated RT-qPCR developed in this study. NoV GII was detected in 11 out of 171 samples with contamination levels from 1.43 to 3.55 $\log_{10}$ copies/g DT. The NoV GII prevalence in Green Mussels was 10%, which was higher than the prevalence in Oriental Hard Clam (7.14%) and Blood Cockle

(2.9%). All NoV-contaminated shellfish were collected from traditional fish markets (Muara Kamal and Cilincing) harvested from Jakarta Bay.

Due to the paucity of relevant data, a deterministic approach was used to estimate the risk of illness due to the consumption of NoV contaminated shellfish from Indonesian markets. In the worst-case scenario where the level of contamination is 8.98×10^3 $\log_{10}$ copies/g DT, boiling for more than 30 min during cooking step can significantly reduce the estimated NoV outbreaks due to shellfish consumption.

Based on the results from the inactivation studies, both inactivation treatments (heat and ClO_2) can be used as control measures to reduce NoV contamination in shellfish. Even though MS2 was more susceptible to heat treatment than NoV, the use of this surrogate in those studies has provided a better understanding on inactivation kinetics and tailing phenomenon in both treatments. Together with the data of NoV exposure or prevalence in shellfish from the markets, the result from the inactivation studies was used to develop a risk assessment that can assist in risk management.

These data provided scientific evidence which can be applied to improve the quality and safety of shellfish production and provide consumer protection from NoV infection in Indonesia. The findings from this study also emphasised the need for regular surveillance in the polluted growing or harvesting areas such as Jakarta Bay, and the application of proper cooking or disinfection to reduce the risks of NoV gastroenteritis from consumption of the contaminated shellfish.

Chapter 1. Literature review

1.1. Introduction

Foodborne disease outbreaks cause serious health problems and are an economic burden in every country. (WHO, 2013) estimated that 2.2 million people die each year due to foodborne and waterborne outbreaks around the world. Many epidemiological studies of foodborne pathogens have shown that bacteria and viruses have the potential to cause serious foodborne illness in humans (Bartsch *et al.*, 2016; Pires *et al.*, 2015; Scallan *et al.*, 2015). In the United States of America they are responsible for 9.4 million episodes of foodborne illnesses per year (Scallan *et al.*, 2011), caused 112,000 DALYs (disability-adjusted life years) (Scallan *et al.*, 2015) and associated with an economic loss of US$10-83 billion per year (Nyachuba, 2010). Diarrhoea and vomiting are the most noticeable symptoms caused by pathogenic foodborne microbes and potentially generate the secondary transmission of the disease through faecal/fomites-oral route and person-to-person transmission (Verhoef *et al.*, 2015).

Among these causative agents, enteric viruses have been associated with high numbers of gastroenteritis outbreaks in infants and the elderly especially at hospital, child care and long term facilities care (LTFC) (Barclay *et al.*, 2014; Bernard *et al.*, 2014; Nic Fhogartaigh & Dance, 2013). Some enteric viruses such as norovirus (NoV) and hepatitis A virus (HAV) have been found in aquatic environments and thus contaminate shellfish (La Bella *et al.*, 2016) and water used for food processing and irrigation (Cook & Richards, 2013). These viruses can generate outbreaks as they can be transmitted with relatively low 'infectious dose' through food or water to humans, or directly from person-to-person (Atmar *et al.*, 2014; Bitler *et al.*, 2013; Hall *et al.*, 2011). Enteric viruses are commonly shed in high numbers in faeces and transferred to fomites in contact with the infected patients, *e.g.*: NoV levels have been reported to range from 10^5 to 10^9 viruses/g faeces (Teunis *et al.*, 2015) and HAV up to 10^9 viruses/g faeces (Kotwal & Cannon, 2014; Tjon *et al.*, 2006).

Although enteric viruses are mostly transmitted person-to-person, food and water are also potential sources of contamination leading to many foodborne outbreaks. Various foods have been reported to be contaminated by viruses and associated with outbreaks, including deli sandwiches (Daniels *et al.*, 2000), salad and produce (Gallimore *et al.*, 2005; Mesquita & Nascimento, 2009; White *et al.*, 1986), raspberries (Le Guyader *et al.*, 2004), frozen strawberries (Hutin *et al.*, 1999), and shellfish (Kohn *et al.*, 1995; Le Guyader *et al.*, 2006; Morse *et al.*, 1986). Other studies also found that contaminated water is responsible for many gastroenteritis outbreaks caused by enteric viruses (Beller *et al.*, 1997; Kukkula *et al.*, 1999) indicating the use of contaminated water for irrigation, aquaculture or drinking purposes. In Australia, outbreaks of HAV occurred in several states during 2009 caused by the consumption of semi dried tomatoes (Donnan *et al.*, 2012), while in 2013 NoV outbreaks were reported in Tasmania associated with the consumption of oysters (Lodo *et al.*, 2014).

Viruses have different structures and behaviours from bacteria. In general, viruses are more than 10 times smaller in size than bacteria with diameters ranging from 25 to 400 nm. Because of their small size, most viruses cannot be observed under the light microscope. Viruses are unable to reproduce and perform metabolic process without their host cell (*i.e.* specific cell type that they can infect and in which they can proliferate). Most of them have a crystalline structure based on a protein shell called a 'capsid' which encloses the DNA or RNA for replication and accessing the host cell (Madigan *et al.*, 2015; Panno, 2011; Prasad *et al.*, 1999). Therefore, because of their relatively simple structure, and particularly the absence of a membrane (*i.e.*, 'non-enveloped' virus) some viruses including human NoV, rotavirus and HAV are more resistant than bacteria from treatments such as chlorination, UV and filtration during conventional wastewater treatment (Corrêa *et al.*, 2012; Duizer *et al.*, 2004; Rzeżutka & Cook, 2004). Unlike the pathogenic bacteria, however, viruses are unable to replicate themselves in the environment due to the lack of a host cell. Therefore, the number of viruses will not increase after shedding from an infected individual and the public health risk will not increase over time as the product moves through the supply chain.

This literature review introduces background information on epidemiology, biology, detection, inactivation, and risk assessment of NoV in food. In addition, information about human enteric viruses relevant to food and shellfish consumption is described to emphasize the importance of human NoV in foods and foodborne outbreaks worldwide.

1.1.1. Human enteric viruses

Enteric viruses that are commonly associated with foodborne and waterborne outbreaks belong to the families *Adenoviridae* (human adenoviruses serotype 40 and 41), *Astroviridae* (human astrovirus types 1 to 8), *Caliciviridae* (NoV & sapoviruses), *Picornaviridae* (aichi viruses, enteroviruses and HAV), *Reoviridae* (rotaviruses) (Bányai *et al.*, 2018; Fong & Lipp, 2005; Le Guyader *et al.*, 2008; Oude Munnink & Van der Hoek, 2016; Thomas *et al.*, 2013). Of these families, *Caliciviridae, Picornaviridae* and *Reoviridae* are mostly found in faeces and fomites from infected people during gastroenteritis outbreaks. *Caliciviridae* and *Picornaviridae* families have a similar morphology and structure, *i.e.,* icosahedral, a non-enveloped RNA virus and similar genome configurations (King *et al.*, 2011). Enteric viruses contaminate food and water through two ways: i) inadequately treated human and animal sewage that contaminates food and water environments and ii) direct contact of food and water with a food handler who has infected by the virus (Gallimore *et al.*, 2005; Maunula & Von Bonsdorff, 2014; Tuladhar *et al.*, 2013).

Numerous food and waterborne outbreaks have been caused by enteric viruses such as NoV, HAV, hepatitis E (HEV), rotavirus, astrovirus and sapovirus (SAV). In USA, Scallan *et al.* (2011) estimated that 59% (5.51 million of a total of 9.4 million) of cases of foodborne illnesses were caused by viruses. Among these viruses, NoV has been estimated as the major cause of viral foodborne illness in USA comprising at least 99% (5.46 million) of the cases, while other enteric viruses compose only less than 1% from the total cases (Scallan *et al.*, 2011). In addition, other studies have also reported the contribution of enteric viruses to foodborne cases worldwide, such as NoV, aichiviruses, rotaviruses, SaV, enteroviruses, astroviruses, and HEV, in Japan (Iritani *et al.*, 2014; Miyashita *et al.*,

2012; Shibata *et al.*, 2015; Usuku *et al.*, 2008), SaV ini Puerto Rico (Hassan-Ríos *et al.*, 2013), NoV, rotaviruses and SaVs in Northern Arabian Gulf (Gallimore *et al.*, 2005), NoV in Sweden, (Hedlund *et al.*, 2000), HAV in the Netherlands and Australia (Donnan *et al.*, 2012; Fournet *et al.*, 2012) and NoV, rotaviruses, and HAV in the USA (Fletcher *et al.*, 2000; Hutin *et al.*, 1999; Noel *et al.*, 1997).

In general, the numbers of viral foodborne cases caused by non-NoV are lower than NoV. This is probably due to several reasons. Firstly, some enteric viruses remain unreported and not necessarily diagnosed as causative of foodborne cases by general practitioners (Maunula & Von Bonsdorff, 2014). Secondly, the availability of vaccines for several enteric viruses such as rotavirus, HAV and HEV may reduce or prevent outbreaks (Nelson *et al.*, 2014; Van Herck *et al.*, 2011; Yen *et al.*, 2011). Thirdly, some viruses such as rotavirus and adenoviruses are childhood disease (Amaral *et al.*, 2015), thus child vaccination program provides a sufficient host-immunity to the viral infection (Braeckman *et al.*, 2012). Lastly, NoV is also known to be persistent in the environment and has a low 'infectious dose', at 18-2,800 viral particles (Rodríguez-Lázaro *et al.*, 2012; Teunis *et al.*, 2008). Combined with high shedding rates of NoV from infected humans, a single infected individual has a potential to transmit and infect hundreds of thousands of people (Pringle *et al.*, 2015). Therefore, the low 'infectious dose' and high shedding rate are presumably the main reasons that NoV is the major enteric virus associated with outbreaks.

As a consequence, NoV is an important issue to be addressed by food safety researchers and health authorities in many countries. Many studies have assessed the risk for consuming food and water contaminated by NoV such as produce (Barker, 2014; Bouwknegt *et al.*, 2015; Laura *et al.*, 2012; Mok *et al.*, 2014), shellfish (Croci *et al.*, 2007; Suffredini *et al.*, 2014) and drinking water (Masago *et al.*, 2006). Although the NoV can now be cultured *in vivo* using stem cell-derived from human enteroids (Ettayebi *et al.*, 2016), however, this cell culture system is still unsuitable as a robust quantification assay for NoV (Ettayebi *et al.*, 2016). Thus, it hampers the development of inactivation models and risk assessment studies. Therefore the use of cultivable NoV surrogates for inactivation studies such as murine norovirus (MNV) (Bozkurt *et al.*, 2014b), feline calicivirus (FCV) (Buckow *et*

al., 2008), virus-like particles (VLPs) (Feng *et al.*, 2011; Koromyslova *et al.*, 2015) and MS2 bacteriophage (MS2) (Bae & Schwab, 2008; D'Souza & Su, 2010) could be alternatives even though their genetic structures are different from human NoV.

1.1.2. Human norovirus

NoV, previously known as Norwalk-like virus (Figure 1-1), causes almost 20% of human gastroenteritis outbreak cases worldwide (Ahmed *et al.*, 2014; Karst *et al.*, 2015). There are three genogroups of NoV (GI, GII and GIV) associated with human gastroenteritis outbreaks (Karst *et al.*, 2015; Zheng *et al.*, 2006). These genogroups are further divided into 33 genotypes based on amino acid sequence diversity in the complete VP1 capsid protein, with 9 genotypes in GI, 22 genotypes in GII and 2 genotypes in GIV (Vinjé, 2015). Of these, only GI and GII genogroups, known as human NoV, are frequently found as contaminants in food and have caused human gastroenteritis through the faecal-oral route (Scallan *et al.*, 2011; Torok, 2013; Yu *et al.*, 2015), especially in raw or uncooked shellfish (Li *et al.*, 2014).

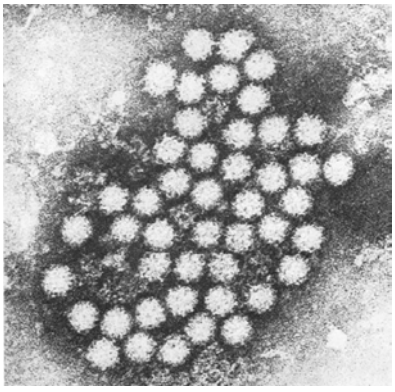


Figure 1-1. Immuno-electron micrograph of NoV in stool samples (reproduced from Kapikian *et al.* (1972)).

Each NoV genogroup has been reported to be specific with respect to binding capability to the host (Tan & Jiang, 2007), environmental persistence (Seitz *et al.*, 2011; Verhaelen *et al.*, 2013) and removal or elimination responses (Cook *et al.*, 2016; da Silva *et al.*, 2007). These differences may influence the epidemiological patterns (Matthews *et al.*, 2012), the distribution in the environment (Hoa *et al.*, 2013) and transmission to the host, especially to humans (Vega *et al.*, 2014). For

example, in a profiling study of NoV genogroups and genotypes during outbreaks, Verhoef *et al.* (2010) showed that although NoV GII was also involed in some foodborne and waterborne outbreaks, NoV GI was more likely to be associated with foodborne cases while NoV Genogroup II including genotype 4 (GII.4) strains were more often related to person-to-person outbreaks. Therefore, the proportion of NoV genotypes associated with foodborne outbreaks could be estimated by analysing NoV outbreak data and genotype profiling from different outbreaks globally (Verhoef *et al.*, 2015).

1.1.3. Structure and biology of norovirus

Human NoV is a small virus, with 23-40 nm in diameter and classified in the family *Caliciviridae* (Vinjé, 2015). NoVs are non-enveloped with icosahedral symmetry composed of 180 protein molecules that form the capsid. The molecules are organised into 90 dimers which have three basic domains, *i.e.*, S, P1 and P2 (Estes *et al.*, 2006). These domains are linked by a flexible hinge. This morphological structure of NoV has been illustrated from the study of three-dimensional structure of recombinant Norwalk virus capsid by Prasad *et al.* (1999) using cryo-image reconstruction and x-ray crystallography (Figure 1-2).

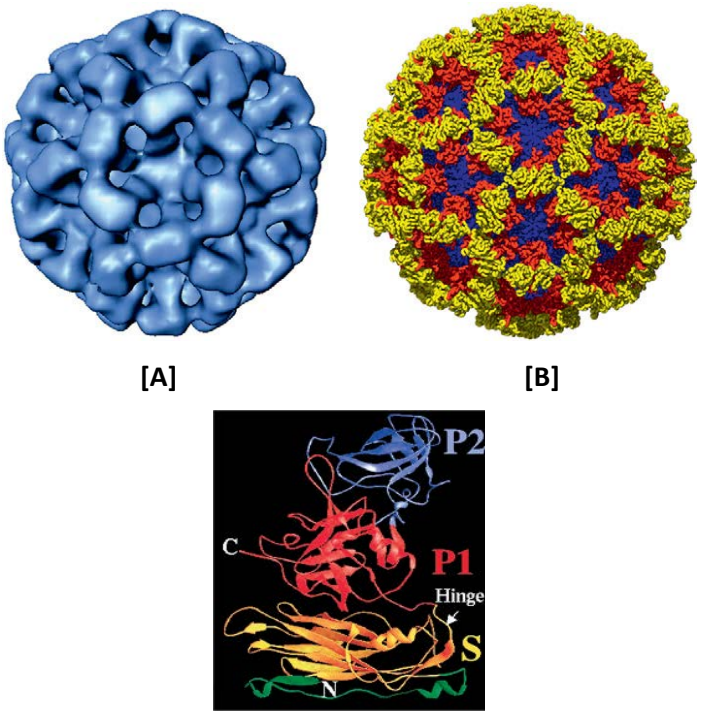


Figure 1-2. Illustration of cryo-image reconstruction (A) and x-ray crystallography (B) of recombinant Norwalk virus capsid structure; and three ribbon-protein domains (C) (reproduced from Prasad *et al.* (1999)).

The genome of human NoV is composed of single-stranded, positive-sense RNA of approximately 7.6 kb length and containing 3 open reading frames (ORFs): ORF1, ORF2 and ORF3 (Atmar *et al.*, 2018). The ORF1 is translated to encode a polyprotein containing six to seven non-structural proteins, including the VPg and the viral RNA-dependent RNA polymerase (RdRp), while the ORF2 and ORF3 are translated from sub-genomic RNA to form two structural proteins during viral replication, the major (VP1) and the minor (VP2) capsid (Karst *et al.*, 2014; Karst *et al.*, 2015; Thorne & Goodfellow, 2014) (Figure 1-3). Generally, the genetic diversity of human NoV is determined from the variability of RdRp and VP1 gene (Kroneman *et al.*, 2013; Stals *et al.*, 2012a; Vinjé *et al.*, 2004; Zheng *et al.*, 2006).

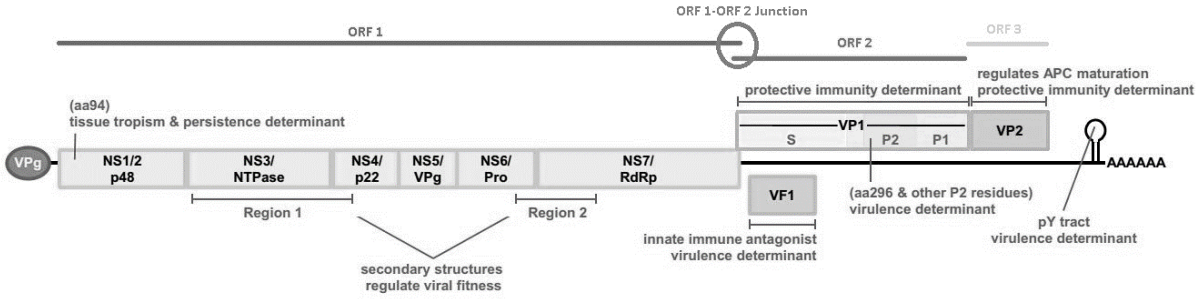


Figure 1-3. The NoV genome (reproduced from Karst *et al.* (2014)).

The ORF1 and ORF2 sequences contain five genomic regions (A, B, C, D and E) that have become the most interesting sequences for detection and genotyping studies (Kroneman *et al.*, 2013; Stals *et al.*, 2012b). These five genomic regions are considered as the most conserved region for GI and GII genogroups (Jothikumar *et al.*, 2005; Kageyama *et al.*, 2003; Loisy *et al.*, 2005; Vinjé *et al.*, 2004), and are widely used for NoV genotyping purpose following single and dual-nomenclature system (Kroneman *et al.*, 2013). Among these, the B and C regions are now commonly used for detection of NoV than theother regions (Le Guyader *et al.*, 2009; Trujillo *et al.*, 2006; Vinjé, 2015). The A and B regions are located at the ORF1 encoding RNA polymerase/RdRp, while region C, D and E are located at the ORF1-ORF2 junction and ORF2 encoding VP1 capsid protein, (Figure 1-4) (Mattison *et al.*, 2009).

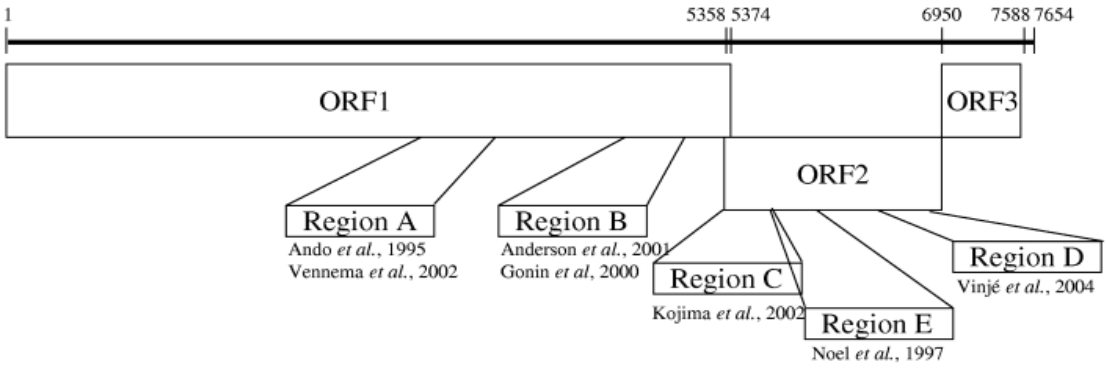


Figure 1-4. Schematic representation of the NoV genome representing five regions frequently used for detection and genotyping study (reproduced from Mattison *et al.* (2009)).

1.1.4. Foodborne norovirus related diseases

A comprehensive study of NoV epidemiology from 1999-2012 by Verhoef *et al.* (2015) reported that person-to-person transmission is the main source of NoV outbreaks and almost 14% of all NoV outbreaks are associated with food as a source of exposure, while the other sources are water and environment. GII.4 was the major causative genotype of NoV outbreaks worldwide being responsible for at least 62% of total NoV cases (Siebenga *et al.*, 2009). This is probably due to the emergence of new variant GII.4 strains every year replacing the previous dominant strains of NoV GII.4 (not other endemic strains) (Siebenga *et al.*, 2009). The high mutation frequency of this strain enhances their ability to bind a wider range of histo-blood group antigens (HBGAs) (White, 2014).

Based on its rapid evolution and immunogenetic response, GII.4 viruses are able to cause gastroenteritis outbreaks in susceptible populations through person-to-person and environmental transmission (Eden *et al.*, 2013; Lindesmith *et al.*, 2012). Non-GII.4 genotypes such as GI.3, GI.6, GI.7, GII.3, GII.6, and GII.12 are more resistant to mutation and only cause gastroenteritis outbreaks *via* food and water transmission route (Vega *et al.*, 2014; White, 2014). Accordingly, several studies have suggested that these genotypes were more consistently the causative agents of waterborne and foodborne outbreaks rather than person-to-person route (Matthews *et al.*, 2012; Vega *et al.*, 2014; Verhoef *et al.*, 2010).

Among the various types of food, produce and shellfish are more susceptible to NoV contamination. Many studies reported that NoV outbreaks were associated with the consumption of contaminated ready-to-eat food such as oyster, clam (Huppatz *et al.*, 2008; Lodo *et al.*, 2014; Morse *et al.*, 1986; Westrell *et al.*, 2010) and fresh produce (Daniels *et al.*, 2000; Gallimore *et al.*, 2005; Mesquita & Nascimento, 2009; Rajko-Nenow *et al.*, 2014). These foods have been indicated to sometimes be grown in, irrigated with and/or processed with NoV-contaminated water, and because they are usually eaten without a proper cooking step, these represent a potential route of human exposure to NoV.

1.1.5. NoV in shellfish

NoVs have been reported to be introduced to water environment by the sewage overflows (Rodríguez *et al.*, 2012) and contaminated marine water (Wyn-Jones *et al.*, 2011; Yang *et al.*, 2012), urban catchments water and estuarine bay (Aw *et al.*, 2009). Due to the presence and persistence of NoV in the water (Cook *et al.*, 2016), shellfish, as a filter feeder animal, are more susceptible to contamination than other seafood products (Lees, 2000). NoV contamination in shellfish has been reported from markets worldwide, such as France (Loutreul *et al.*, 2014), Thailand (Kittigul *et al.*, 2016), Italy (Terio *et al.*, 2010) and Australia (Symes *et al.*, 2007). Other studies have also reported the presence of NoV in shellfish harvested from Portugal (Mesquita *et al.*, 2011), UK (Lowther *et al.*, 2012), Italy (Croci *et al.*, 2007), France (Le Guyader *et al.*, 2009), the Netherlands (Boxman *et al.*, 2006), Australia (Brake *et al.*, 2014), Japan (Maekawa *et al.*, 2007) and India (Umesha *et al.*, 2008). Although the contamination has been widely reported, the risk assessment of NoV in shellfish is still rare and partially performed, especially in Asian countries. In Indonesia particularly, the NoV prevalence in shellfish from Indonesian fish markets or harvesting area is not yet available. Consequently, acquiring knowledge for risk assessment of NoV in shellfish has become important to provide better understanding of NoV outbreaks worldwide including in Indonesia to aid the development of preventive strategies against future outbreaks.

1.2. Bivalve molluscan shellfish

1.2.1. Biology of shellfish

Bivalve molluscs are soft bodied animals that belong to the Bivalve class. The soft bodies are protected by two opposed shell valves composed of calcium carbonate. This class is the second largest class within the molluscs and consists of 7,500 species. Generally, species identification of bivalves is based on their colour, shape and marking on the shell. More than 80% of bivalves live in the ocean and these organisms are important element of marine and freshwater habitats (Gosling,

2003, 2015). Some of these bivalves including mussels, oysters, scallops and clams (Figure 1-5) are also called as ‘shellfish’ in aquaculture and fishery studies.

Figure 1-5. Shellfish from Bivalvia Class (reproduced from Gosling (2015))

Shellfish are highly modified molluscs, including modification of the gill function to entrap food particles from the aqueous environment. It enables shellfish to feed efficiently in aqueous environments. This feeding system, known as ‘filter feeding’, is the most efficient system of ciliary feeding in sea animals (Gosling, 2003). Shellfish are able to filter large volumes of water from their environment and accumulate different types of suspended food particles, and pathogenic bacteria and viruses (Le Guyader *et al.*, 2013; Lees, 2000), in their gills. Moreover, these accumulated viruses are concentrated in DT by HBGA-like for carbohydrate ligand molecules which may enhance the bioaccumulation process (Maalouf *et al.*, 2011). Hence many studies have proposed that DT can be used for detection, quantification and isolation of NoV from shellfish.

1.2.2. Shellfish production

There are five major groups of bivalve molluscs which are commonly consumed by humans and grown/harvested and sold commercially*:* mussels, oysters, scallops, clams and cockles. In 2010,

world shellfish production was 10% of the total global fisheries production, with 14.6 million tons of production. 12.9 million ton of this production originated from aquaculture activities, consisting of 38% clams, cockles and ark shells; 35% oysters; 14% mussels and 13% scallops. The high demand for shellfish in the global market, at US$ 2.1 billion in 2009, triggered high production of shellfish worldwide. Scallops were the most important shellfish species in international markets and accounted for 46% of the total shellfish production (Karunasagar, 2014). However, the increasing scale of shellfish production should be matched by increasing the public awareness about the risk of raw shellfish consumption.

1.2.3. Shellfish in Indonesia

As shown in Figure 1-6, a statistical report from Food Agriculture Organization (FAO) (2015) showed that, during the period 2007 to 2010, shellfish production in Indonesia increased rapidly from 10,000 to 70,000 metric tonnes. In 2011, the proportion of shellfish consumption was only around 15% of total shellfish production, while the rest was utilised for non-consumption purposes such as pearl oyster. Up to 2007 the trend of shellfish consumption in Indonesia was relatively stable with the average of 10,000 metric tonnes per year (FAO, 2015). Shellfish consumed in Indonesia are mainly produced from aquaculture activities including fresh, brackish and marine water (Nurdjana, 2006). The major commodities are Green Mussel (*Perna viridis*), Oriental Hard Clams (*Meretrix lusiora*), Bamboo Clam (*Ensis directus*), Blood Cockle (*Anadara granosa*), and Feather Cockle (*Anadara antiquata*).

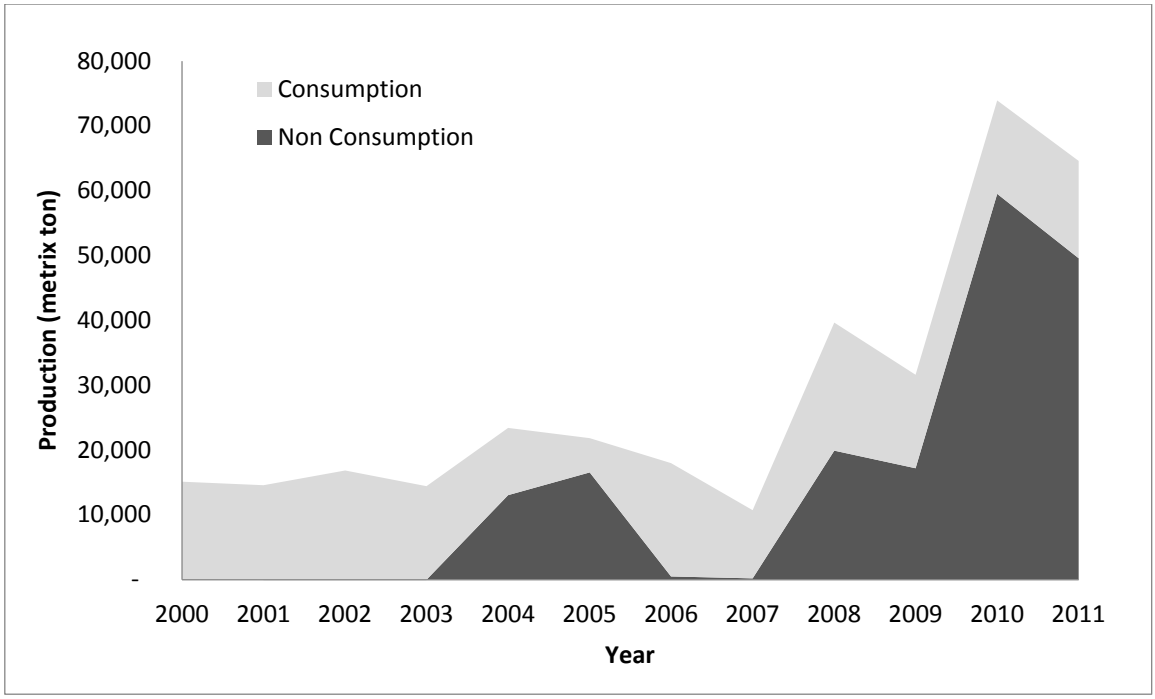


Figure 1-6. Indonesia shellfish production from 2002-2011 (reproduced from FAO (2015))

People in Indonesia usually consume cooked shellfish, such as boiled, steamed or fried. Eating raw shellfish, such as oysters, has not been widely introduced. It has started to be advertised in restaurants in several big cities such as Jakarta, Surabaya and Denpasar, especially for tourists or 'foreigners'. In this case, the oyster's quality is strictly controlled, and the raw materials are mostly imported from Eastern Asian countries such as Taiwan, Korea and Japan that employ shellfish sanitation programs.

Decree of the Indonesian Minister of MAF no. KEP.17/MEN/2004 regulates the Indonesian shellfish sanitation system and aims to ensure the production of safe shellfish from Indonesia for local and export markets. The regulation assists different parties that play roles in the shellfish production system, including the shellfish farmers, processors, distributors, and the competent authorities who monitor and control the application of sanitation system. The central and local competent authorities are responsible for conducting monitoring and routine surveillance on the application of the Sanitation Standard Operational Procedure (SSOP), Good Manufacturing Practices (GMP), Good Hygienic Practices (GHP), as well as the integrated quality management program based on HACCP, in every aspect of the shellfish production system. The authorities are also responsible for laboratory

testing to ensure the shellfish conformity with safety and quality requirements set in the Indonesian National Standard (SNI) No. 3460.1; 3460.2; and 3460.3 (BSN, 2009).

The sanitation system also includes regulation for the shellfish farms across Indonesian waters. The farm's locations are regularly assessed, then the water qualities are recorded and routinely monitored to determine the suitability of the locations to be used to grow the shellfish. Based on the microbiological quality of the water and the possibility of pollution in the area due to the natural cause and anthropogenic activities, the shellfish growing areas are classified into permissible areas, permissible areas with certain condition, limited areas and off-limit areas. Shellfish farming activities are prohibited in the off-limit areas. These areas are characterised by a high level of faecal contamination, an exceeding level of PSP toxin, or the areas that have not been assessed for the sanitation compliance.

Another part of the Indonesian shellfish sanitary system relates with the post-processing activities, such as handling, collection, processing and distribution. For live shellfish, the transportation and distribution should be done in a temperature-controlled vehicle, to avoid the shellfish quality loss and their survival. Furthermore, a repeat circulation system with sterilised water may be used for depuration purposes. The standard quality and safety requirements set in the decree for live shellfish and its processed products for direct consumption are presented in Table 1-1.

Table 1-1. Standard quality for live shellfish and its processed products for direct consumption (MMAF Indonesia, 2004)

Parameters	Requirement	Method of analysis
Visual characteristics	Eggshells clean from manure, giving reaction to knock, contain normal intravulval liquid	Visual observation
Faecal coliform/*Eschericia coli*	Coliform < 300 MPN/100 g and *E. coli* < 230 MPN/100 g of shellfish meat, based on 5 tubes	Most Probable Number (3 dilutions)
Salmonella	Absence in 25 g of shellfish meat	
Total PSP content	Must not exceed 80 µg/100 g of shellfish meat	Bioassay test
PSP (diarrhetic shellfish poisoning)	Negative	Bioassay test
ASP (amnestic shellfish poisoning)	Must not exceed 20 µg/100 g of domoic acid	HPLC
Mercury (Hg)	Must not exceed 0.5 mg/kg	
Lead (Pb)	Maximum of 1.5 mg/kg	
Cadmium (Cd)	Maximum of 1 mg/kg	

1.3. Detection and quantification methods for noroviruses

To improve the safety of shellfish in the European countries, the European Food Safety Authority (EFSA) has published a scientific opinion that contains recommendations to the European Council for the establishment of regulations to control NoV contamination in oysters. One of the recommendations is to investigate the levels of NoV contamination in shellfish which requires a suitable method of identification and quantification (EFSA Panel on Biological Hazards (BIOHAZ), 2012). Accordingly, many studies have detected and quantified NoV in shellfish, in water as well as sewage using methods such as conventional RT-PCR (Baert *et al.*, 2007; Kageyama *et al.*, 2003; Kojima *et al.*, 2002; Vinjé *et al.*, 2004), RT-qPCR (Greening & Hewitt, 2008; Le Guyader *et al.*, 2009; Suffredini *et al.*, 2014), enzyme-based colorimetric assay (Batule *et al.*, 2018), immunoassay and LAMP.

Among these methods, RT-qPCR has become a gold standard assay for both detection and quantification (ISO, 2013; ISO, 2017), and it is widely used in NoV quantification studies (Kirby & Iturriza-Gómara, 2012; Le Guyader *et al.*, 2006; Vinjé, 2015). However, RT-qPCR may fail to

distinguish between infectious and non-infectious viruses in the sample because the assay will quantify the RNA from both infectious and non-infectious viral particles. This drawback can lead to misinterpretation of viral inactivation data for food quality control (Ceuppens *et al.*, 2014). As a consequence, sample pre-treatments to differentiate infectious from non-infectious viral RNA and modification of RT-qPCR methods are required to provide a better analysis.

1.3.1. Primer sequences for detection, genotyping and quantification of NoV by RT-qPCR

Since the beginning of 2000's, the use of both conventional and RT-qPCR methods to detect NoV has increased rapidly. Many highly sensitive primer sets have been designed to detect both NoV GI and GII such as in food, environmental and clinical samples as shown in Table 1-2. Most of the primers target the sequences of ORF1, ORF1-ORF2 junction and ORF2 (GenBank accession no. X86557, nt 4997 to 5108) for GII detection, and sequences from the ORF1-ORF2 junction and ORF2 (GenBank accession no. M87661, nt 5271 to 5385) for GI detection, (Figure 1-7), and only few primers target different sequences of ORF2 in region D of GI (nt 5354 to 6914) and GII (nt 6432 to 6684) (Kong *et al.*, 2015; Vinjé *et al.*, 2004).

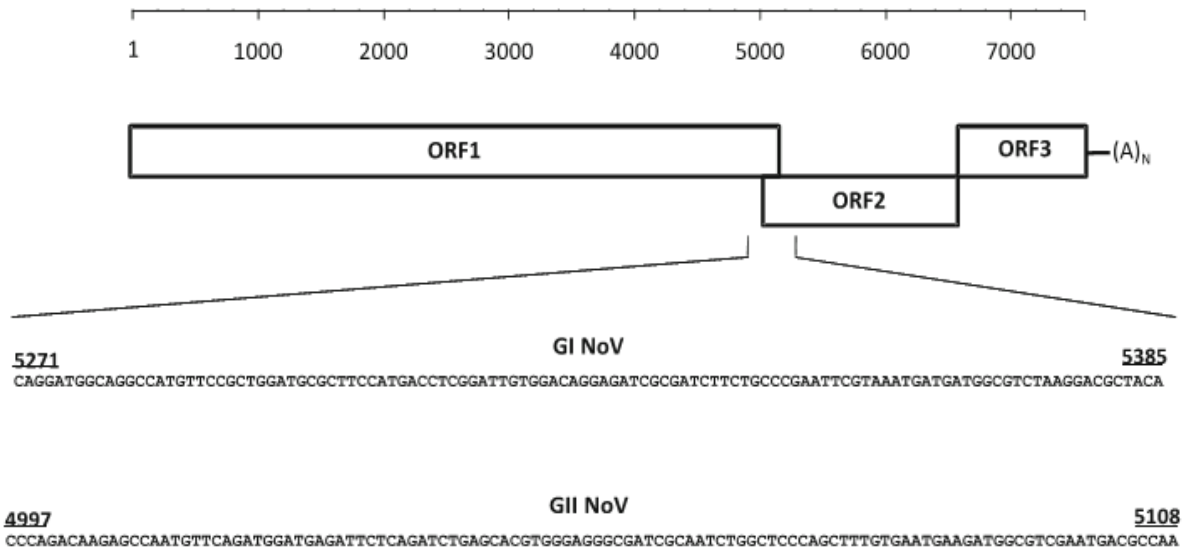


Figure 1-7. The target sequences of ORF 1, ORF1-ORF2 junction and ORF2 for the detection of NoV GI and GII genogroups (reproduced from Stals *et al.* (2012b)).

Table 1-2. Set of primer sequences for detection (D), genotyping (G) and quantification (Q)of NoV GI and GII by RT PCR assay

NoV Geno-group	Primer	Sequences	Polar-ity	Melting Temp (°C)	Product Length (bp)	Location	Type of assay* (D/G/Q)[1,2]	References
GI	G1SKF	5'-CTG CCC GAA TTY GTA AAT GA-3'	+	49.7	329	ORF1-ORF2 junction & ORF 2	D/G[1,2]	(Kojima *et al.*, 2002)
	G1SKR	5'-CCA ACC CAR CCA TTR TAC A-3'	-	51.1	329	ORF1-ORF2 junction & ORF 2	D/G[1,2]	(Kojima *et al.*, 2002)
	COG1F	5'-CGY TGG ATG CGN TTY CAT GA-3'	+	55.9	84	ORF1-ORF2 junction	Q[1,2]	(Kageyama *et al.*, 2003)
	COG1R	5'-CTT AGA CGC CAT CAT CAT TYA C-3'	-	53	84	ORF1-ORF2 junction	Q[1,2]	(Kageyama *et al.*, 2003)
	G1FFa	5'-ATH GAA CGY CAA ATY TTC TGG AC-3'	+	55.3	596	ORF1-ORF2 junction & ORF 2	G[1,2]	(Kageyama *et al.*, 2004)
	G1FFb	5'-ATH GAA AGA CAA ATC TAC TGG AC-3'	+	51.7	596	ORF1-ORF2 junction & ORF 2	G[1,2]	(Kageyama *et al.*, 2004)
	G1FFc	5'-ATH GAR AGR CAR CTN TGG TGG AC-3'	+	60.6	596	ORF1-ORF2 junction & ORF 2	G[1,2]	(Kageyama *et al.*, 2004)
	G1SKR	5'-CCA ACC CAR CCA TTR TAC A-3'	-	51.1	596	ORF1-ORF2 junction & ORF 2	G[1,2]	(Kageyama *et al.*, 2004)
	Cap A	5'-GGC WGT TCC CAC AGG CTT-3'	+	54.2	177	ORF2	G[1]	(Vinjé *et al.*, 2004)
	Cap B1	5'-TAT GTT GAC CCT GAT AC-3'	-	57.6	177	ORF2	G[1]	(Vinjé *et al.*, 2004)
	Cap B2	5'-TAT GTI GAY CCW GAC AC-3'	-	59.1	177	ORF2	G[1]	(Vinjé *et al.*, 2004)
	NIFG1F	5'-ATG TTC CGC TGG ATG CG-3'	+	55.9	92	ORF1-ORF2 junction	Q[1,2]	(Miura *et al.*, 2013)
	QNIF4	5'-CGC TGG TAG CGN TTC CAT-3'	+	55	86	ORF1-ORF2 junction	Q[1,2]	(da Silva *et al.*, 2007)
	NV1LCR	5'-CCT TAG ACG CCA TCA TCA TTT AC-3'	-	56	86	ORF1-ORF2 junction	Q[1,2]	(Svraka *et al.*, 2007)
	NKIF	5'-GTA AAT GAT GAT GGC GTC TAA-3'	+	50.3	305-314	ORF2	D/G[1]	(Kong *et al.*, 2015)
	NKI-F2	5'-GAT GGC GTC TAA GGA CGC-3'	+	55.8	305-314	ORF2	D/G[1]	(Kong *et al.*, 2015)
	NKIR	5'-ACC CAD CCA TTR TAC ATY TG-3'	-	50.8	305-314	ORF2	D/G[1]	(Kong *et al.*, 2015)
	MON 432	5’-TGG ACI CGY GGI CCY AAY CA-3’	+	57.2	213	ORF1	D/G[1,2]	(Richards *et al.*, 2004)
	MON 434	5’GAA SCG CAT CCA RCG GAA CAT-3’	-	56.3	213	ORF1	D/G[1,2]	(Morillo *et al.*, 2012)

Table 1-2. Continued…

NoV Geno-group	Primer	Sequences	Polar-ity	Melting Temp (°C)	Product Length (bp)	Location	Type of assay* (D/G/Q)[1,2]	References
GII	G2SKF	5'-CNT GGG AGG GCG ATC GCA A-3'	+	57.6	343	ORF1 & ORF2	D/G[1,2]	(Kojima *et al.*, 2002)
	G2SKR	5'-CCR CCN GCA TRH CCR TTR TAC AT-3'	-	62.4	343	ORF1 & ORF2	D/G[1,2]	(Kojima *et al.*, 2002)
	G2FBa	5'-GGH CCM BMD TTY TAC AGC AA-3'	+	57.9	479	ORF1 & ORF2	Q[1,2]	(Kageyama *et al.*, 2004)
	G2FBb	5'-GGH CCM BMD TTY TAC AAG AA-3'	+	55.9	479	ORF1 & ORF2	Q[1,2]	(Kageyama *et al.*, 2004)
	G2FBc	5'-GGH CCM BMD TTY TAC ARN AA-3'	+	57.9	479	ORF1 & ORF2	Q[1,2]	(Kageyama *et al.*, 2004)
	G2SKR	5'-CCR CCN GCA TRH CCR TTR TAC AT-3'	-	62.4	479	ORF1 & ORF2	D/G[1,2]	(Kageyama *et al.*, 2003; Kojima *et al.*, 2002)
	Cap C	5'-CCT TYC CAK WTC CCA YGG-3'	+	54.2	253	ORF2	G[1]	(Vinjé *et al.*, 2004)
	Cap D1	5'-TGT CTR STC CCC CAG GAA TG-3'	-	57.6	253	ORF2	G[1]	(Vinjé *et al.*, 2004)
	Cap D3	5'-TGY CTY ITI CCH CAR GAA TGG-3'	-	59.1	253	ORF2	G[1]	(Vinjé *et al.*, 2004)
	COG2F	5'-CAR GAR BCN ATG TTY AGR TGG ATG AG-3'	+	57.6	97	ORF1-ORF2 junction	Q[1,2]	(Kageyama *et al.*, 2003)
	QNIF2	5'-ATG TTC AGR TGG ATG AGR TTC TCW GA-3'	+	57.4	88	ORF1-ORF2 junction	Q[1,2]	(Loisy *et al.*, 2005)
	JJV2F	5'-CAA GAG TCA ATG TTT AGG TGG ATG AG-3'	+	55.6	97	ORF1-ORF2 junction	D/Q[1,2]	(Boxman *et al.*, 2009; Jothikumar *et al.*, 2005)
	COG2R	5'-TCG ACG CCA TCT TCA TTC ACA-3'	-	56.6	97	ORF1-ORF2 junction	D/Q[1,2]	(Jothikumar *et al.*, 2005; Kageyama *et al.*, 2003)
	NVG2flux1	5'-ATG TTY AGR TGG ATG AGR TTY TC-3'	+	55.3	88	ORF1-ORF2 junction	Q[1]	(Nordgren *et al.*, 2008)
	NVG2flux2	5'-GGG AGG GCG ATC GCA ATC T-3'	+	55.4	51	ORF1-ORF2 junction	Q[1]	(Bucardo *et al.*, 2017)
	MON 431	5'-TGG ACI AGR GGI CCY AAY CA-3'	+	54.9	213	ORF1	D/G[1,2]	(Richards *et al.*, 2004)
	MON 433	5'GAA YCT CAT CCA YCT GAA CAT-3'	-	52.4	213	ORF1	D/G[1,2]	(Morillo *et al.*, 2012)

Note: ** (D=detection; G=genotyping; Q=quantification assay)*

[1] Primer has been used in the PCR assay for clinical samples

[2] Primer has been used in the PCR assay for various food matrices including shellfish samples

The ORF1 and ORF1-ORF2 junction encoding both RdRp and major capsid (V1) is a sufficiently conserved region for NoV GI and GII detection (Kageyama *et al.*, 2003; Stals *et al.*, 2012b). Therefore, the use of primers designed from these sequences were able to detect 95-99% of GII genogroups from confirmed positive samples with sensitivity from <10 to 10^4 genomic copies (Kojima *et al.*, 2002; Vinjé *et al.*, 2004) but in some cases, these primers are less able to detect emerging variants of GII.4 genotypes (Stals *et al.*, 2012b). To improve the detection of these new variants there is a need to design or develop new primer sets from different sequence regions based on new strains isolated and identified from new outbreaks.

Based on the reverse transcription reaction prior to PCR assay, there are two types of RT-qPCR method, *i.e.*, one and two-step RT-qPCR. Both methods are comparable in terms of specificity, efficiency and reliability. Although, two-step RT-qPCR has been commonly applied in NoV quantification for clinical and food samples, the one-step method could be a promising method for routine analysis because it is quicker, easier, and less expensive (Al-Shanti *et al.*, 2009; Kirby & Iturriza-Gómara, 2012). In addition, the use of a single reaction tube in the one-step method could minimise sample cross-contamination, and the consequences of inaccurate pipetting during the reverse transcription (Hanaki *et al.*, 2014; Vinjé, 2015). Therefore, one-step RT-qPCR has been applied for standard quantification of NoV (ISO, 2013; ISO, 2017), such as in inactivation and surrogate studies in shellfish, water and faecal samples (Coudray-Meunier *et al.*, 2015; Fuentes *et al.*, 2014; Jothikumar *et al.*, 2005; Miura *et al.*, 2013).

A method which can detect multiple NoV genogroups (GI, GII and GIV) simultaneously has been developed using multiplex RT-qPCR and it has been suggested to be useful for the rapid screening of NoV in food and water (Miura *et al.*, 2013). However, single RT-qPCR produces a better sensitivity than multiplex, especially when detecting low numbers of NoV, probably due to the use of more probes (Niwa *et al.*, 2014) and the number of genomes to be amplified (Stals *et al.*, 2009) by multiplex RT-qPCR. Furthermore, single RT-qPCR is more suitable to be used than multiplex assays in inactivation studies where only one NoV genogroups is being studied at a time.

1.3.2. Sample pre-treatment in NoV inactivation studies to differentiate infectious/non-infectious viruses

Published risk assessment studies on NoV in produce and ready-to-eat food (Bouwknegt *et al.*, 2015; Mokhtari & Jaykus, 2009; Stals *et al.*, 2015), drinking water and environment (Masago *et al.*, 2006; Mok *et al.*, 2014; Victoria *et al.*, 2010) and shellfish products (Ventrone *et al.*, 2013) have quantified both infectious and non-infectious viruses without differentiation. Since only infectious particles of NoV can infect humans, the number of quantified viral particles in those studies might not represent the amount of infectious virus in the samples.

In recent years, the application of a pre-treatment step prior to RT-qPCR assay has been widely studied to quantify infectious norovirus (Gyawali *et al.*, 2019; Knight *et al.*, 2012). The infectivity of virus can be determined by its genom stability or capsid integrity (Knight *et al.*, 2012). Thus, the mechanism of pre-treatment step is based on two different processes, *i.e.*, the capability of the specific substances and chemical to disrupt the genom of infectious viral particles (damaged capsid), or to bind the infectious viral particles (undamaged capsid and genom) (Knight *et al.*, 2012).

Pre-treatments with photoactivable dyes (propidium monoazide (PMA), PMAxx™, PEMAX™ and EMA) (Gyawali & Hewitt, 2018; Kim & Ko, 2012; Oristo *et al.*, 2018; Parshionikar *et al.*, 2010), Porcine Gastric Mucin (PGM) (Li *et al.*, 2013; Ye *et al.*, 2014), *in situ* capture (Wang *et al.*, 2014) and RNase (Richards *et al.*, 2012; Ronnqvist *et al.*, 2013) prior to RT-qPCR assay have been applied to evaluate the efficacy of NoV inactivation treatment by quantifying the infectious NoV. The use of RT-qPCR pre-treated with RNase are, to date, the most reliable and promising methods to be applied because they are more efficient and economically affordable than the other methods.

RNase is known to be effective as a pre-treatment to quantify infectious viral particles of NoV surrogates such as MNV, MS2 and HAV (Nuanualsuwan & Cliver, 2003; Rodríguez *et al.*, 2009). It is able to distinguish infectious and non-infectious viral particles through the evaluation of capsid and cell membrane integrity during nucleic acid extraction prior to RT-qPCR assay (Soto-Munoz *et al.*,

2014; Yang & Griffiths, 2014). The basic principle of this pre-treatment is the degradation of RNA from inactive bacteria and non-infectious viruses which lack of cell membrane or viral capsid integrity, respectively (Knight *et al.*, 2012).

However, the efficacy of RNase to degrade viral RNA depends on the different inactivation methods and target viruses in the assay. For example, RNase was more effective when used as a pre-treatment for measuring infectious viral particles treated by UV than high temperature due to different in the mechanism of genomic structure degradation by the different treatments (Bhattacharya et al., 2004). In addition, an inactivation study of human NoV which is previously known as a snow mountain virus (SMV), reported that RNase is less effective than PMA (Escudero-Abarca et al., 2014). Each virus has different capsid structure and ionic strength, thus they have different capabilities to survive changes in temperature, pH and ionic strength in the suspension during inactivation experiments (Knight et al., 2012).

Another alternative enzyme group that has potential to be used as pre-treatment are restriction enzymes (REs). Molloy and Symons (1980) reported the ability of eight REs to cleave DNA in an RNA-DNA substrate and amongst them, HaeIII and TaqI have also been shown to cleave the RNA strand of this heteroduplex substrate. A further study by Murray *et al.* (2010) identified the cut-site or sequence-specific site of TaqI enzyme to cleave DNA and RNA strands as T/CGA, while other REs also identified to have similar ability were AvaII (cut site G/GWCC, W=A or T), AvrII (cut site C/CTAGG) and BanI (cut site G/GYRCC, Y=C or T, R=A or G). That study also showed that these enzymes cleave RNA-DNA and DNA-DNA substrate at the same phosphodiester bonds. However, the efficiency of these enzymes to hydrolyse RNA strands from heteroduplex substrates is at least two orders of magnitude less than the hydrolysis of DNA from homoduplex (DNA-DNA) substrate. Despite this, there is the potential for using TaqI as a pre-treatment enzyme prior to RNA extraction to eliminate free RNA from non-infectious viruses. Study to evaluate the efficacy of this enzyme as a pre-treatment prior RT-qPCR is necessary, especially for the viral quantification in water, faecal, food matrices including shellfish as there is no available data about its efficacy until now.

1.4. Inactivation of human NoV in shellfish

Apart from the NoV quantification method development that has been described above, many studies have also investigated the treatment to reduce or eliminate NoV using depuration (Polo *et al.*, 2014), high pressure processing (HPP) (Ye *et al.*, 2014), high temperature (Ahmed *et al.*, 2013; Escudero-Abarca *et al.*, 2014; Ettayebi *et al.*, 2016; Li *et al.*, 2013; Wang & Tian, 2014), electron beam and gamma irradiation (Feng *et al.*, 2011; Praveen *et al.*, 2013), 70% ethanol, UV, chlorine and other chemical sanitisers (Belliot *et al.*, 2008; Costantini *et al.*, 2018; D'Souza & Su, 2010; Ronnqvist *et al.*, 2013). Some of these treatments, such as UV and application of disinfectants might be ineffective to eliminate viral particles bioaccumulated inside the shellfish because the treatments cannot penetrate the viral particles inside the tissue. High temperature is considered as the best treatments which resulted in a higher log reduction of viral particles either in artificially contaminated shellfish or in buffered media (Araud *et al.*, 2016; Bozkurt *et al.*, 2014b; Kingsley *et al.*, 2014). In addition, chlorine-based compounds also caused high reduction of infectious viral particles (D'Souza & Su, 2010), thus it can be applied as a potential disinfectant or sanitizer agent to reduce viral particles which contaminated food by cross-contamination during processing and handling (FAO & WHO, 2009). Since shellfish in Indonesia is commonly consumed in a cooked form, the application of high temperature treatment may not affect the consumer preference. During post-harvest step, the retailers in Indonesian generally wash the tissue or the whole body of shellfish using clean water or water containing disinfectant (WWF-Indonesia, 2015). Therefore, high temperature treatment and chlorine-based disinfectants could be the most effective way to reduce and to eliminate NoV in naturally-contaminated and in cross-contaminated shellfish from Indonesian fish markets, respectively.

1.4.1. NoV inactivation studies using surrogates

Despite the significant impact of NoV in foodborne disease, the major limitation to the study of this virus is its uncultivable nature (Cannon *et al.*, 2006; Patel *et al.*, 2008). To overcome this, some

studies on NoV inactivation have proposed the use of a cultivable NoV surrogate such as FCV, murine noroviruses (MNV-1), tulane virus (TuV) or MS2 (Cromeans *et al.*, 2014; Farkas *et al.*, 2010; Flannery *et al.*, 2013; Kingsley *et al.*, 2007) which share or have similar biochemical and genetic properties to NoV (Jiang *et al.*, 1993; Kniel, 2014; Wobus *et al.*, 2006). These surrogates are amongst the most common surrogates used in inactivation studies of NoV in different environments, such as water, seafood and produce (Bae & Schwab, 2008; Belliot *et al.*, 2008; Bozkurt *et al.*, 2014b; Cannon *et al.*, 2006; Dawson *et al.*, 2005). Since the proposed viral surrogates can be grown in a cell system or small animals (Baert *et al.*, 2008; Wobus *et al.*, 2006), they can be used in routine clinical assays (Kniel, 2014). However, the presence of less structural variations in surrogates compared to the NoV necessitates the use of multiple surrogates in one study (Kniel, 2014).

Bacteriophages are a group of viruses that infect bacterial cells and share common physical, biological and chemical characteristics with some mammalian viral pathogens. When viable host is absent in an environment, bacteriophage cannot replicate themselves. Moreover, their host specificity is limited to bacteria which means they can only infect bacteria and not mammalian cells, so they do not pose a risk for humans. Also, they are cheap and generally easy to maintain in the laboratory (Tufenkji & Emelko, 2011). Therefore, bacteriophage, such as MS2, has been used as NoV surrogates in studies of enteric viruses.

MS2 is a ssRNA bacteriophage with capsid and known as one of the simplest viruses (Tufenkji & Emelko, 2011). Compared to other types of phage, MS2 is the most robust model virus to be used in a viral aerosol study and produced similar results when detected using qPCR and plaque assay (Turgeon *et al.*, 2014). These properties support the use of MS2 in inactivation and removal studies of NoV in different types of food including water (Bae & Schwab, 2008; Hornstra *et al.*, 2011), fresh produce (Dawson *et al.*, 2005), pork (Brandsma *et al.*, 2012) and shellfish (Love *et al.*, 2010).

1.4.2. Chlorination

For many years, chlorination, also known as "chlorine-containing disinfectants" treatment (FAO & WHO, 2009), has been known as an effective treatment to reduce the number of pathogenic bacteria and viruses in contaminated food. Sodium hypochlorite ($NaClO_2$) as an oxidizing agent is widely used as a disinfectant in food processing plants because it is cheap and easily applied (Fonseca, 2006). Moreover, another less harmful chlorine-containing compound such as chlorine dioxide (ClO_2) treatment can be an alternative as it has been legally approved in the US for use as an anti-microbial agent in food processing (Gómez-López *et al.*, 2009).

Chlorine is a strong oxidizing compound which is able to destroy viral RNA (O'Brien & Newman, 1979) and bacterial cell membranes (Venkobachar *et al.*, 1977). At an appropriate level, this compound can be directly added into water for drinking (Kitajima *et al.*, 2010) and washing raw food products such as vegetable (Singh *et al.*, 2002), fruit (Chen & Zhu, 2011) and poultry carcasses (Nagel *et al.*, 2013; Sarjit & Dykes, 2015) to reduce the level of pathogenic viruses and bacteria.

In NoV inactivation studies, chlorination has successfully reduced the number of the virus (Kim *et al.*, 2012; Kingsley *et al.*, 2014; Kitajima *et al.*, 2010). These studies reported that chlorination of 0.5 (free chlorine), 189 and 5,000 (total chlorine) ppm were able to reduce NoV by 3.64, 4.14 and 5.26 log_{10} respectively. In contrast with those studies, a study by Duizer *et al.* (2004) suggested that 300 ppm total chlorine was ineffective to reduce the number of NoV in the suspension. Factors that may contribute to the chlorination efficacy are pH, temperature and the presence of organic matter during inactivation (Hirneisen *et al.*, 2010; Kingsley *et al.*, 2014; Morino *et al.*, 2009; Tung *et al.*, 2013). As the RT-PCR assay, which may not be able to distinguish between infectious and non-infectious virus was been used in this study, the different efficacy of chlorination to reduce NoV might be result of overestimation of infectious NoV.

1.4.3. High temperature treatment

The use of high temperature treatment (also known as 'heat treatment') to inactivate microorganisms is widely used food preservation technique. In the food industry, there are four types of heat treatment: pasteurisation, sterilisation, canning and blanching (Teixeira, 2015). In the meat and fish industries, sterilisation and canning are the most popular treatments. The study of high temperature treatment in shellfish industries has been done since 30 years ago by Millard *et al.* (1987). That study evaluated double boiling or cooking at 85-100°C to inactivate HAV and poliovirus (PV) during shellfish processing. Using a radioimmunofocus assay, this method was successfully confirmed to inactivate both viruses. Another study by Hewitt and Greening (2006) also confirmed the efficacy of heating at 90°C for 90 s to inactivate viral particles in mussel.

More recent studies showed that high temperature treatments ranging from 50-80°C for 0.21-20 min exposure were able to reduce NoV and other NoV surrogates in shellfish (Araud *et al.*, 2016; Bozkurt *et al.*, 2014b; Croci *et al.*, 2012). Other studies also showed the ability of high temperature to reduce NoV and its surrogates in different matrixes, such as berries (Butot *et al.*, 2009), water and milk (Hewitt & Greening, 2006) and PBS (Li *et al.*, 2012; Topping *et al.*, 2009; Wang & Tian, 2014). However, the efficacy of heat treatment to reduce the NoV depends on the temperature, time exposure, type of matrix and the initial titers of virus used in the experiments (Arthur & Gibson, 2015). Also NoV shows less susceptible to heat treatment than their surrogates (Knight *et al.*, 2016), therefore the use of the most heat-resistant surrogate is considered (Arthur & Gibson, 2015).

1.4.4. Mathematical modelling on virus inactivation

In the microbial inactivation, changes in the environment due to high temperature or mild inactivation treatment such as chlorination, may lead to a log-linear reduction of cell numbers or a shouldering and tailing expressions (Tamplin, 2005). In the linear phase the decimal reduction time (D value) is defined as the reduction rate or the time needed to inactivate 90% of the initial

population, while, the z value is defined as the changes in temperature needed to produce 90% change in the reduction rate (D value) (Barer, 2012; Tamplin, 2005).

Predictive modelling in food microbiology is used to describe the growth, survival, inactivation as well as the metabolic activities of the microorganisms (Buchanan & Whiting, 1997). It can be categorised based on different approaches, such as the microbial responses toward certain treatment (growth, survival, and inactivation model), mechanistic or empirical model, and the three-tier classification (primary, secondary, and tertiary model). A mechanistic model relies on an *a priori* knowledge of different factors that influence the behaviour of microorganisms, while an empirical model uses experimental data from different sets of conditions (Buchanan & Whiting, 1997; Caffi *et al.*, 2007). Furthermore, Buchanan (1993) defined the three-tier classification of model as follows, *i.e.* a primary model that mathematically describes the microbial responses (growth or survival) towards certain conditions as a factor of time, a secondary model that further describes the effect of environmental factors on the microbial growth and survival; and a tertiary model that combines primary and secondary models into a computer program or software.

The empirical model has been widely used in the modelling of microbial inactivation, including viral inactivation. The first-order kinetic model is a simple linear model assuming that the levels of cells/virus survival during treatment decrease exponentially over time of exposure. A survival curve is obtained by plotting the logarithmic number of survival cells/viruses against the lethal dose received and it is independent to the size of the original population (Barer, 2012). The first-order kinetic model has been used in studies to predict the effect of thermal processing (Buckow *et al.*, 2008; Deboosere *et al.*, 2004a; Isbarn *et al.*, 2007; Pecson *et al.*, 2009) and other mild treatments including HPP (Isbarn *et al.*, 2007) and chlorination (Thurston-Enriquez *et al.*, 2003, 2005) on viral inactivation. The first-order kinetic model can be described in Equation 1-1 and 1-2 (Erkmen & Bozoglu, 2016; IFT, 2000; Moats, 1971), and the D value can be calculated from the slope value of linear regression (Equation 1-3), as below :

$$\frac{dN}{dt} = -kN$$ Equation 1-1.

$$\ln\left[\frac{N}{N_0}\right] = -kt$$ Equation 1-2.

$$\mathrm{D} = -\frac{1}{s}$$ Equation 1-3.

where:

$\frac{dN}{dt}$ = the rate of viral death

N = the number of virus (PFU/ml) at time (t)

N_0 = initial number of virus (PFU/ml)

t = time (h or min)

k = inactivation rate constant

D = 1 $\log_{10}$ reduction (h or min) at time (t)

s = slope of linear regression

This model assumes that each bacterial cell/virus has equal resistance towards the treatments, thus the death from inactivation occurs to each bacterial cell (Erkmen & Bozoglu, 2016; Moats, 1971). However, the first-order kinetic theory does not take into account the initial lag in the death rate (Moats, 1971), while some viral inactivation studies showed that the viral survival curves have shouldering and tailing phenomena on the beginning and end of the curve (Araud *et al.*, 2016; Chen *et al.*, 2005; Sigstam *et al.*, 2014). The shouldering and/or tailing phenomena usually occur when using a high concentration of initial cells and/or at mild heat or lower temperature treatment (Geeraerd *et al.*, 2000; Tamplin, 2005), or when a subpopulation of virus is resistant to the disinfectant (Sigstam *et al.*, 2014) or have a low probability of lethal hit by a water molecule during thermal inactivation (Casolari, 1998). Therefore, the viral inactivation curves often do not follow the linear model assumptions. In this case, the non-linear models such as Weibull and biphasic models are used (Araud *et al.*, 2016; Bozkurt *et al.*, 2014b; Sigstam *et al.*, 2014).

The Weibull model, in particular, has successfully estimated virus survival from different treatments. For example, inactivation of HAV in buffered cell culture treated with HPP (Grove *et al.*, 2009), MNV-

1 treated with HPP (Kingsley *et al.*, 2007), HAV in heat-treated oyster (Lee *et al.*, 2015), HAV in heated blue mussel homogenate (Bozkurt *et al.*, 2014b), HAV, TV, MNV-1 and RV in heated oyster tissue (Araud *et al.*, 2016), and FCV in pressurised and heated culture media (Chen *et al.*, 2005). In some of those studies, the Weibull was compared with the linear (first order kinetic) model and was observed to perform better. The Weibull model is described by the following equation:

$$log \frac{N}{N_0} = -bt^n \qquad \text{Equation1-4.}$$

where:

N = the number of virus (PFU/ml) at time (t)

N_0 = initial number of virus (PFU/ml)

b = the slope factor

t = time (h or min)

n = the scale factor

This model assumes that viral inactivation occurs as probabilities and that the inactivation curve is the cumulative form of distribution of lethal events (Erkmen & Bozoglu, 2016; Kingsley *et al.*, 2006). When applying the Weibull model, the D value is usually determined from the linear portion of the curve (Chen *et al.*, 2005).

Another non-linear model that commonly used to describe the inactivation model of bacteria or virus is biphasic model (Cerf, 1977; de Roda Husman *et al.*, 2009). This model is based an assumption that two subpopulation of cells/virus having different levels of resistance to treatments are present in the bacterial/virus population during inactivation (Cerf, 1977; Humpheson *et al.*, 1998). Therefore, this model produces two linear curves representing the survival of each subpopulation over the time exposure thus generate two D value, *i.e.*, D initial and D tailing. The D values of this model can be generated from both linear regression equation. The Biphasic model (derived from Cerf (1977)) is described by following equation (Geeraerd *et al.*, 2005):

$$log10(N) = log10(N_0) + log10\left(f.e^{-kmax_1.t} + (1-f).e^{-kmax_2.t}\right)$$ Equation 1-5.

where:

N = the number of virus (PFU/ml or copies/ml) at time (t)

N_0 = initial number of virus (PFU/ml or copies/ml)

t = time (h or min)

f = fraction of the initial population in major subpopulation

$kmax_1$ and $kmax_2$ = specific inactivation rate of two population (phase 1 and phase 2, respectively)

1.5. Risk assessment of human NoV in shellfish

Studies on method development and inactivation treatments of NoV have been widely investigated, however risk assessment of the virus in food and shellfish which comprehensively incorporates these studies are still limited. For instance, the available risk assessment studies used RT-qPCR method without pre-treatment which is unable to distinguish between infectious and non-infectious NoV (Bouwknegt *et al.*, 2015; Masago *et al.*, 2006; Mok *et al.*, 2014; Stals *et al.*, 2015; Suffredini *et al.*, 2014). Hence, the level of exposure and prevalence data produced from these studies may not represent the real risk exposure of NoV. Therefore, combining the modified detection methods which can differentiate infectious and non-infectious virus together with inactivation treatments will enhance the risk prediction in a risk assessment study.

In addition, published risk assessment studies of NoV have been conducted for developed countries such as European countries, Japan, and Australia (Bouwknegt *et al.*, 2015; Masago *et al.*, 2006; Stals *et al.*, 2011; Suffredini *et al.*, 2014), which in general have different shellfish eating behaviour compared to people in Indonesia.

1.6. Thesis objectives

As the consumption of shellfish continues to increase in Indonesia, the development of an accurate risk assessment, using a reliable quantification method of NoV and based on the specific eating

behaviour of shellfish in Indonesia, is needed to estimate the exposure and risk of NoV in shellfish in Indonesia.

The overall objective of this research is to estimate the risk of NoV which might contaminate shellfish in Indonesian fish markets. By applying RT-qPCR with enzyme pre-treatment in NoV inactivation studies and in NoV prevalence study, a more comprehensive risk assessment will be developed. There are four aims which contribute to this thesis:

1. To evaluate the application of RT-qPCR pre-treated with enzymes for NoV inactivation studies.
2. To compare the inactivation kinetic of NoV and its surrogate (MS2 bacteriophage) treated by heating and chlorine dioxide.
3. To determine the efficacy of high temperature treatment on NoV and MS2 reduction in buffer and in bioaccumulated-shellfish (inside the tissue).
4. To determine the efficacy of chlorine dioxide as disinfectant to reduce NoV and MS2 in buffer and in artificially-contaminated shellfish (in the surface)
5. To determine the NoV prevalence in raw shellfish from Indonesian fish markets.

The risk assessment will provide scientific-based recommendations for the Indonesian government and the related stakeholders. The recommendations can be applied to improve the quality and safety of shellfish industries as well as provide consumer protection from foodborne outbreaks related to NoV.

Chapter 2. Improving molecular quantification of infectious MS2 bacteriophage: A norovirus surrogate for inactivation studies

2.1. Introduction

NoV is considered to be one of the major causes of foodborne disease globally causing almost 20% of all cases of acute gastroenteritis worldwide (Ahmed *et al.*, 2014; Karst *et al.*, 2015), or an estimated 120 million diarrhoeal cases and 5,000 deaths globally in 2010 (Havelaar *et al.*, 2015), mostly in developing nations. In USA, NoV is estimated to cause 5.46 million foodborne diseases each year (Scallan *et al.*, 2011). NoV transmission to humans is predominantly by person to person, followed by food and environmental transmission (Glass *et al.*, 2009; Verhoef *et al.*, 2015). Shellfish, soft berries and leafy salads are food types that commonly associated with NoV contamination in food (FAO & WHO, 2008).

A major limitation to study NoV is the difficulty to quantify the viral particles using the previously developed cell culture system (Cannon *et al.*, 2006; Ettayebi *et al.*, 2016; Patel *et al.*, 2008). Consequently, RT-qPCR has become a standard diagnostic tool or reference method for NoV detection and quantification (Glass *et al.*, 2009; ISO, 2013; ISO, 2017; Ushijima *et al.*, 2014). However, the RT-qPCR assays that are available for detection of total nucleic acid, cannot distinguish between infectious and non-infectious NoV (Knight *et al.*, 2012): the ribonucleic acid (RNA) from non-infectious virus remains detectable but undistinguishable by PCR assay even though the virus has lost its infectivity (Richards, 1999). Therefore, NoV quantification by RT-qPCR assay could over-estimate the abundance of NoV and hence the risk of illness to humans from NoV in contaminated food, water or environmental samples.

As previously described in section 1.3 and 1.3.2, many studies have investigated the application of pre-treatment step to improve the quantification of infectious viral particles and the utilization of viral surrogates to evaluate its efficacy. For example, RNase is reported to be effective as a pre-

treatment in RT-qPCR to quantify only infectious viral particles of NoV (Richards *et al.*, 2012) and its surrogates such as MNV (Ronnqvist *et al.*, 2013), FCV and HAV (Nuanualsuwan & Cliver, 2002). However, the efficacy of the RNase pre-treatment appears to depend on the type of virus inactivation process, especially under harsh inactivation conditions (Pecson *et al.*, 2009; Topping *et al.*, 2009). From those studies, RNase pre-treatment significantly reduced the amplification of RNA from non-infectious viral particles by heat treatment. Without further inactivation of RNase following the pre-treatment, however, RNase may remain in the sample during extraction resulting in the degradation of RNA from infectious viral particles. This may contribute to under-estimation of viral abundance by PCR assay.

The strategies to overcome this problem are to eliminate and to inactivate residual RNase activity, for example, by using of guanidinium thiocyanate and 2-mercaptoethanol during nucleic acid extraction (Chomczynski & Sacchi, 2006); or adding an RNase inhibitor (RNasin) (Nuanualsuwan & Cliver, 2002; Yang & Griffiths, 2014); or heating the samples (Johnson, 1996) prior to nucleic acid extraction. However, heating the samples prior to RNA extraction is not common practice as it may affect the RNA integrity (Brisco & Morley, 2012) and that results in inaccurate quantification of the PCR assay. As an alternative to RNase as pre-treatment, the use of different enzymes such as restriction enzymes is being considered, mainly because the application of these enzymes is cheaper than RNase+RNasin and safer than the application of 2-mercaptoethanol during nucleic acid extraction. Molloy and Symons (1980) and Murray *et al.* (2010) showed that some restriction enzymes such as HaeIII and TaqI were able to cleave DNA and RNA strands. Hence these enzymes have potential to be used to disrupt free genomic RNA from inactivated viral particles.

Some authors have proposed the use of cultivable NoV surrogates such as FCV and TV (Cromeans *et al.*, 2014; Farkas *et al.*, 2010), MNV (Cromeans *et al.*, 2014; Kingsley *et al.*, 2007), FRNA bacteriophages (Flannery *et al.*, 2013; Hartard *et al.*, 2016) and MS2 bacteriophage (MS2) (Hornstra *et al.*, 2011) to explore NoV inactivation kinetics. MS2, belongs to genus *Levivirus* of family

Leviviridae, and is a non-harmful cultivable virus which has a similar structure to NoV and has been frequently used as a NoV surrogate (Brié *et al.*, 2016; Hornstra *et al.*, 2011; Sherchan *et al.*, 2014; Turgeon *et al.*, 2014). Therefore, the use of MS2 as a NoV surrogate together with the application of enzymatic pre-treatments, such as RNases and TaqI, could be a promising approach for quantification methods and for inactivation studies of NoV.

In this study, we examined the performance of an RT-qPCR method with RNase and TaqI pre-treatments to quantify MS2 bacteriophage as a NoV surrogate and to demonstrate the use of these methods for the quantification of the NoV surrogate after high temperature and chlorine dioxide (ClO_2) treatments.

2.2. Materials and methods

2.2.1. MS2 bacteriophage stock production

MS2 bacteriophage (MS2) was cultivated as previously described by Bae and Schwab (2008) with the following modification. MS2 (ATCC® 15597-B1™) purchased from In Vitro Technologies (Australia) was inoculated into host *E. coli* strain K12 (culture collection of Tasmanian Institute of Agriculture) at a ratio of approximately 10^7 PFU of MS2 per 10^{10} CFU of *E. coli* cells in 100 ml of Luria-Bertani (LB) broth (Oxoid, UK; CM0996) containing 10 mM added calcium chloride ($CaCl_2$) (Sigma Aldrich, USA) and 0.1% glycine (Sigma Aldrich, USA). The mixture was incubated at 37°C with continuous shaking for 8 to 12 h until bacterial lysis occurred. Ten ml of chloroform (Sigma Aldrich, USA) was then added to the suspension and incubated for a further 10 min at 37°C. The culture was then centrifuged at 5,000 x *g* for 10 min to remove *E. coli* cells and cell debris, and the virus-containing supernatant was recovered as MS2 stock. The MS2 stock was serially filtered through 0.45 and 0.22 µm pore-size low-protein-binding membrane filters (Millipore, Germany) and stored at -80°C. The concentration of MS2 in the stock was determined as described in Section 2.2. The plaque assays and RT-qPCR results of infectious MS2 stocks at concentrations from 10^0 to 10^7 PFU/µl were compared and analysed by

linear regression using Microsoft Excel® (Microsoft, USA), to determine the correlation coefficient (R^2 value).

2.2.2. Quantification of MS2

2.2.2.1. Plaque assay

MS2 were quantified using a double layer agar method (EPA, 2001) with modification, using *E. coli* strain K12 as the host strain and LB^+ as the culture media. In brief, 3 ml aliquots of semi-solid LB^+ agar (LB broth containing 0.7% (w/w) agar, 10 mM $CaCl_2$ and 0.1% glycine) were pre-warmed at 45°C in a shaking water bath. Then, 100 µl of exponential phase *E. coli*, containing approximately 10^{6-7} CFU/ml, was added as a host. One hundred µl of serially diluted MS2 stock were added to the pre-warmed semi-solid LB^+ agar (~ 45°C) and then poured into pre-warmed (~ 45°C) 90 mm Petri plates containing solid LB^+ agar (LB broth + 1.5 % (w/w) agar + 10 mM $CaCl_2$ + 0.1% glycine). After 18-24 h of incubation at 37°C, MS2 were quantified by counting the semi-transparent plaques formed on the LB^+ agar plates. This assay only quantified the presence of MS2 between 2 to 200 PFU per plate. Therefore, the theoretical limit of quantification (LOQ) of this assay is 2 PFU per 100 µl of sample that is equivalent to 1.30 $\log_{10}$ PFU/ml.

2.2.2.2. RT-qPCR development

a. Plasmid and standard production

For absolute quantification, a plasmid standard was constructed by cloning nucleotides from 1470 to 2000 of MS2 sequences (GenBank accession no. NC_001417) as previously described by Gentilomi *et al.* (2008), with the TOPO II Kit (Invitrogen, USA). The fragment produced had 531 bps length. Plasmid was purified using a plasmid purification kit (MO BIO, Australia) following the manufacturer's recommended procedures and quantified using a Nano Drop 8000 (Thermo Scientific, USA). Plasmid was linearized by PCR using M13 primers provided with the TOPO II Kit (Invitrogen, USA). The PCR product had a length of approximately 774 bps encompassing 243 bps of original M13 sites plus 531 bps of inserted MS2 gene. The product was then purified using a PCR

purification kit (MO BIO, Australia) and quantified using a Fragment Analyzer™ (Advanced Analytical Technology Inc., USA). Standard concentrations for each plasmid used were 10,000,000; 1,000,000; 100,000; 10,000; 1,000; 100; 10; and 1 copies per µl. Copy number of the linearized plasmid was calculated using Equation 2-1.

$$\text{Number of copies (molecules)} = \frac{\text{X ng} * 6.02221 \times 10^{23} \text{ molecules/mole}}{(\text{N} * 660 \text{ g/mole}) * 1 \times 10^{9} \text{ng/g}} \qquad \text{Equation 2-1}$$

Where:

X = amount of amplicon (ng)

N = length of dsDNA amplicon

660 g/mole = average mass of 1 bp dsDNA

b. RNA extraction

Genomic RNA was extracted from liquid samples of MS2 by the acid-guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (2006) with modifications. Specifically, two hundred µl of liquid sample were mixed with 1 ml denaturing solution (containing 4M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% *N*-laurosylsarcosine and 0.1M 2-mercaptoethanol) and gently shaken for 15 sec. Then 0.1 ml of 2M sodium acetate pH 4.0, 1 ml of water-saturated phenol, 0.2 ml of chloroform/isoamyl alcohol (49:1) were added and the tubes were shaken vigorously for 10 sec. The samples were incubated at 4°C for 15 min and centrifuged for 20 min at 10,000 *x g* at 4°C. The aqueous phase was transferred to new microtubes containing 1 ml of cold isopropanol (approximately -20°C) (Sigma Aldrich, USA) and incubated at -20°C for at least 1 h. The RNA pellet was precipitated by centrifugation for 20 min at 10,000 *x g* at 4°C. After discarding the supernatant, cold 70% ethanol (approximately -20°C) was added to the pellet and centrifuged for 10 min at 10,000 *x g* at 4°C. The supernatant was removed from the tubes to isolate the RNA pellet. After air drying the pellet for 10 min at room temperature (15-25°C), the RNA was dissolved in 50 µl of DEPC-treated Tris-EDTA (TE) buffer pH 7.2.

c. Quantification of MS2 with one-step RT-qPCR

RT-qPCR was conducted using PowerSYBR® Green RNA-to-CT™1-Step Kit (Applied Biosystem, USA) on a Rotor Gene 3000 (Corbett Research, Australia). Primers used in this assay were designed from MS2 sequences for nucleotides 1733 – 1804 f (GenBank accession no. NC_001417) analysed using Primer-BLAST NCBI software. The primer sequences were 5'-GCCGGCCATTCAAACATGAG-3' (forward) and 5'-CGAGAGAAAGATCGCGAGGAA-3' (reverse).

PCR thermal condition were as follows: initial holding at 48°C for 30 min and 95° for 10 min; followed by 45 cycles of denaturation at 95°C for 15 sec; annealing at 55°C for 30 sec; elongation at 72°C for 1 min and final extension at 72°C for 7 min (Gentilomi *et al.*, 2008). The length of the PCR product amplified from this assay was 92bp. To assess the specificity of PCR product, negative controls using RNA from bacteria and melt curve assays were conducted following the recommended procedures for the Rotor Gene 3000 (Corbett Research, Australia).

2.2.3. Preliminary experiment

This preliminary experiment was done in triplicate to confirm the efficacy of RT-qPCR without enzymatic pre-treatment in MS2 inactivation by heating and chlorination. MS2 suspension was heat-treated at 72° or treated with 0.5 ppm of chlorine dioxide (ClO_2) (Zychem, Australia). For high temperature treatment, MS2 stocks were heated at 72°C using the methods described by Nuanualsuwan and Cliver (2002) with modification. In brief, MS2 stocks were added to the pre-heated (72°C) 2 ml microtubes containing 900 µl PBS to a final concentration of 10^{10} PFU/ml. The samples were heated at 72°C for 15, 30, and 60 min in a water bath. The ClO_2 treatment was performed in a 25°C water bath. Appropriate volumes of 10 ppm ClO_2 were added to 900 µl MS2 in PBS stocks (10^{10} PFU/ml) to reach final concentrations of 0.5 ppm ClO_2 and incubated for 15, 30 and 60 min. After incubation, 10 µl of 1% (w/v) sodium thiosulfate was added to the samples which were incubated for another 10 min to neutralise the oxidising effect of ClO_2. Samples from both heat and

chlorine dioxide treatments were analysed for MS2 both by plaque assay and RT-qPCR performed as described in Sections 2.2.2.1 and 2.2.2.2, respectively.

2.2.4. Development of pre-treatment for RT-qPCR

Ten ml of MS2 suspension at a final concentration of 10^7 - 10^8 PFU/ml was heat–inactivated at 60°C for 120 min. This treatment was done to obtain two sub-populations of viruses *i.e.*, infectious and non-infectious viruses so that, RNA from non-infectious viral particles was present in the suspension. The heated MS2 were pre-treated with RNase, RNase followed by RNasin (RNase+RNasin), or TaqI enzyme. All enzymatic pre-treatments and no pre-treatment (control) were done in triplicate. The RNase+RNasin pre-treatment was carried out as described by Yang and Griffiths (2014) but modified by adding a 4 µl aliquot containing 10 mg/ml of RNase A (Sigma Aldrich, Germany) to 150 µl of virus extract and incubating at 35°C for 30 min. Then, 10 µl of RNasin (40 units/µl) (Promega, USA) was added to the sample and incubated for 30 min at 37°C. For the TaqI pre-treatment, 10 µl of TaqI enzyme (20 units/µl; NEB, USA) was added to 150 µl aliquots of virus extract and incubated at 60°C for 30 min. Three control treatments were included: unheated MS2 without pre-treatment, unheated MS2 with RNase+RNasin pre-treatment and heated MS2 without enzyme pre-treatment. MS2 RNA was extracted and assayed in triplicate as described in Section 2.2. Results were analysed using Analysis of Variance (ANOVA) and Tukey Test post-hoc analysis by SigmaPlot 12.0 Version (Systat Software, USA).

2.2.5. Application of pre-treatment RT-qPCR for inactivation studies

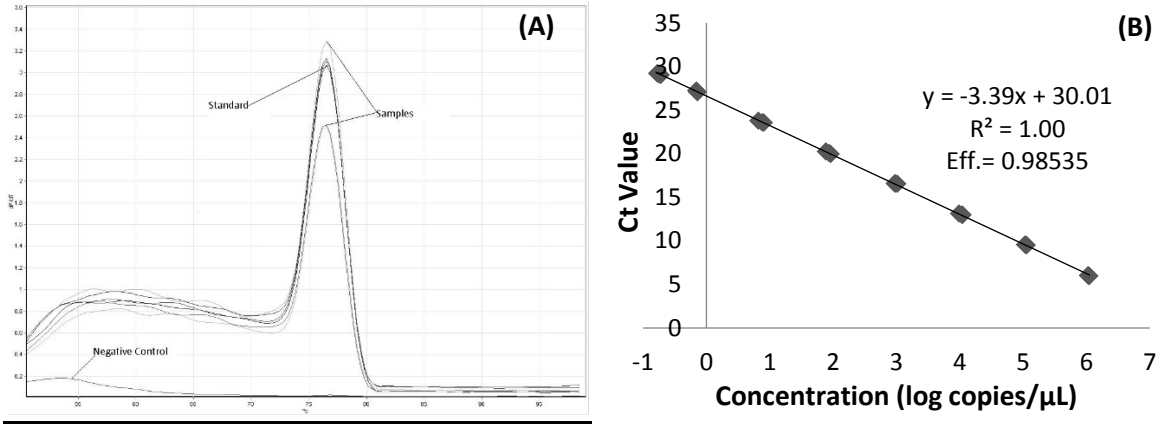
High temperature or chlorination treatments were applied to MS2 suspensions in the inactivation study. The heat treatments were carried out as previously described in section 2.2.3, with modification of time exposure. MS2 stock was added to the pre-heated 2 ml microtubes containing 900 µl PBS to a final concentration of 10^8 PFU/ml. The samples were heated at 72°C for 2.5, 5, 10, 20 and 40 min in a water bath. The ClO_2 treatment was performed in a 25°C water bath. Appropriate volumes of 100 ppm ClO_2 were added to the 10^8 PFU/ml MS2 in PBS stocks to reach final

concentrations of 1, 2, 4, 8 and 16 ppm ClO_2. After 5 min of incubation, 10µl of 1% (w/v) sodium thiosulfate was added to the samples and incubated for another 10 min to neutralise the oxidising effect of chlorine dioxide. The infectious MS2 from both inactivation treatments were assayed in triplicate by plaque assay and the modified RT-qPCR as described in Sections 2.2 and 2.3. Prior to nucleic acid extraction, all samples (including control, heated and chlorine dioxide treated samples) were pre-treated using RNase followed by RNasin.

2.3. Results

2.3.1. The correlation between plaque assay and RT-qPCR

The melt curve analysis showed that the RT-qPCR reaction generated a single peak. Moreover, the genomic RNA from the negative control was not amplified during the PCR reaction. This indicates that the assay only amplified the specific target gene of MS2 and that no non-specific amplification was detected (Figure 2-1A).



**Ct value: a fractional number of cycles where the PCR kinetic curve reaches a user or program-defined threshold amount of fluorescence (Schefe et al., 2006).*

Figure 2-1. Melt curve analysis of the standard and samples (A); and standard curve MS2 plasmid from RT-qPCR assay generated from Rotor Gene 3000 (B)

To quantify the MS2 bacteriophage by RT-qPCR, a standard curve was generated from the linearized MS2 plasmid at concentrations from 10^0 to 10^7copies/µl. The RT-qPCR was found to be less sensitive than the plaque assay with a limit of quantification (LOQ) of 4.46 copies/reaction or 4.46 copies/25

µl (≈ 2.25 log_{10} copies/ml), while the theoretical LOQ of plaque assay is 1.30 log_{10} PFU/ml. The calculated PCR efficiency of the assay was 98% with a slope value of -3.39 and a high correlation of R^2=1.00 (Figure 2-1B).

The correlation between the RT-qPCR and plaque assay were evaluated using only infectious MS2 from unheated stock culture. A high correlation (R^2=0.9978, P<0.001) with a slope value of 0.9938 and an intercept value of -0.13 was obtained (Figure 2-2). From the regression equation, the result from RT-qPCR can be extrapolated to PFU/µl of MS2 where 1 log_{10} copies/µl is equal to 1.14 log_{10} PFU/µl.

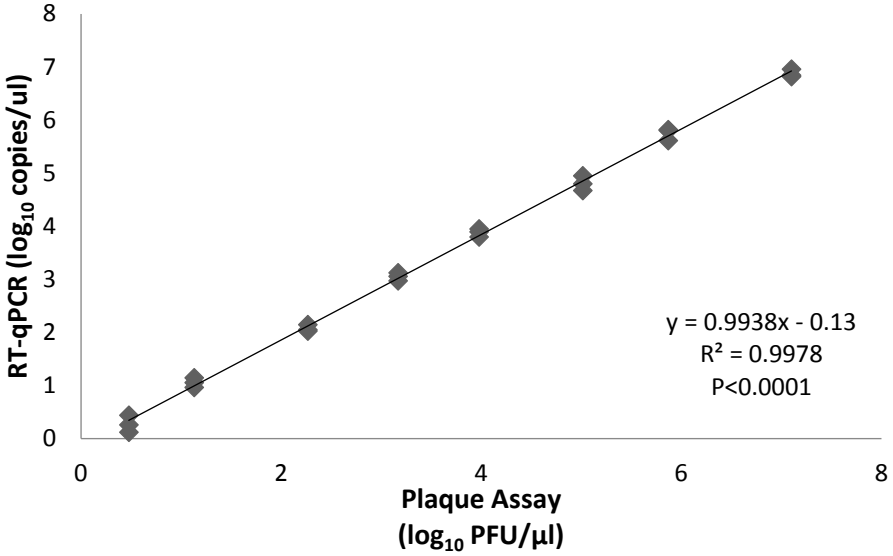


Figure 2-2. The linear correlation between plaque assay and RT-qPCR on the quantification of infectious MS2

2.3.2. Effect of different pre-treatments on the quantification of mixtures of infectious and non-infectious MS2

In the preliminary study, MS2 was treated with high temperature and ClO_2 to obtain a mixture of both infectious and non-infectious MS2. The result from plaque assays showed that heating at 72°C for 15 to 60 min reduced the level of infectious MS2 by 4-9 log_{10} PFU/ml (Figure 2-3A), while ClO_2 at a concentration of 0.5 ppm from 15 to 60 min had no significant (P>0.05) effect on MS2 reduction (Figure 2-3B). In comparison to the plaque assay, the result of RT-qPCR without pre-treatment prior

to nucleic acid extraction showed over-quantification of the infectious MS2 after heating at 72°C for 15-60 min. The RT-qPCR result was approximately 1-6 log_{10} PFU/ml higher than the plaque assay after the heat-treatment (Figure 2-3A), while after the ClO_2 treatment the RT-qPCR assay showed a similar result to plaque assay (Figure 2-3B).

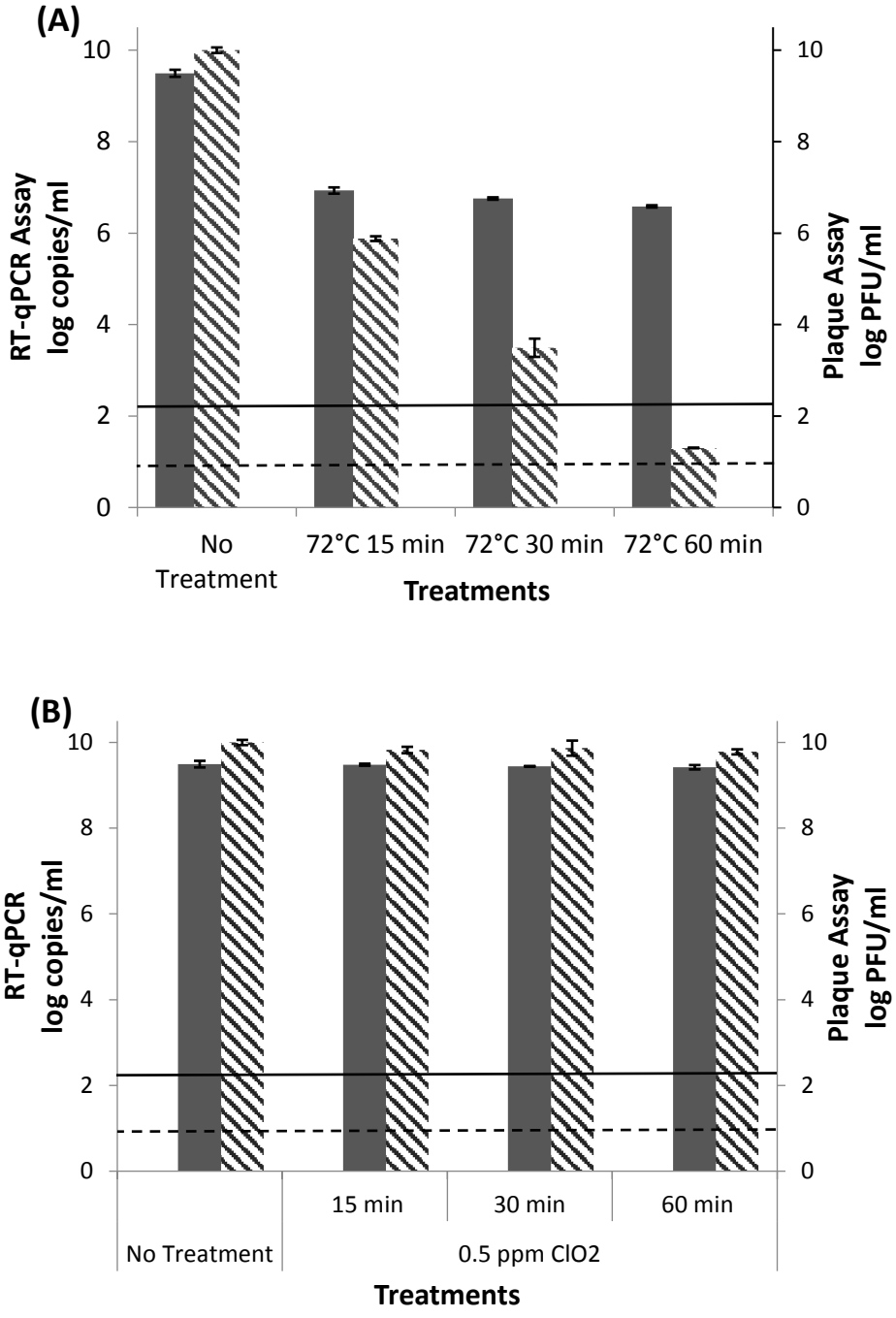


Figure 2-3. Comparison of RT-qPCR with no pre-treatment (■) and the plaque assay (▨) on the quantification of infectious MS2 after heat treatment at 72°C (A) and chlorination with 0.5 ppm of ClO_2 (B) with LOQ of RT-qPCR (—) and plaque assay (- -).

To try to prevent the over-quantification of infectious MS2 by the RT-qPCR assay due to the presence of genome fragments from non-infectious viruses, enzymatic pre-treatment with RNase, RNase+RNasin or TaqI was applied prior to RNA extraction. MS2 that had been pre-treated with RNase, RNase+RNasin or TaqI were analysed using both RT-qPCR and plaque assays. The result of RT-qPCR pre-treated with RNase+RNasin produced no significant difference ($P>0.05$) compared to plaque assays for the quantification of infectious MS2 the heat treatment (Figure 2-4). In contrast, the RT-qPCR pre-treated either with RNase alone or TaqI produced a significantly different ($P<0.001$) result compared to the plaque assay in the quantification of infectious MS2the heat treatment.

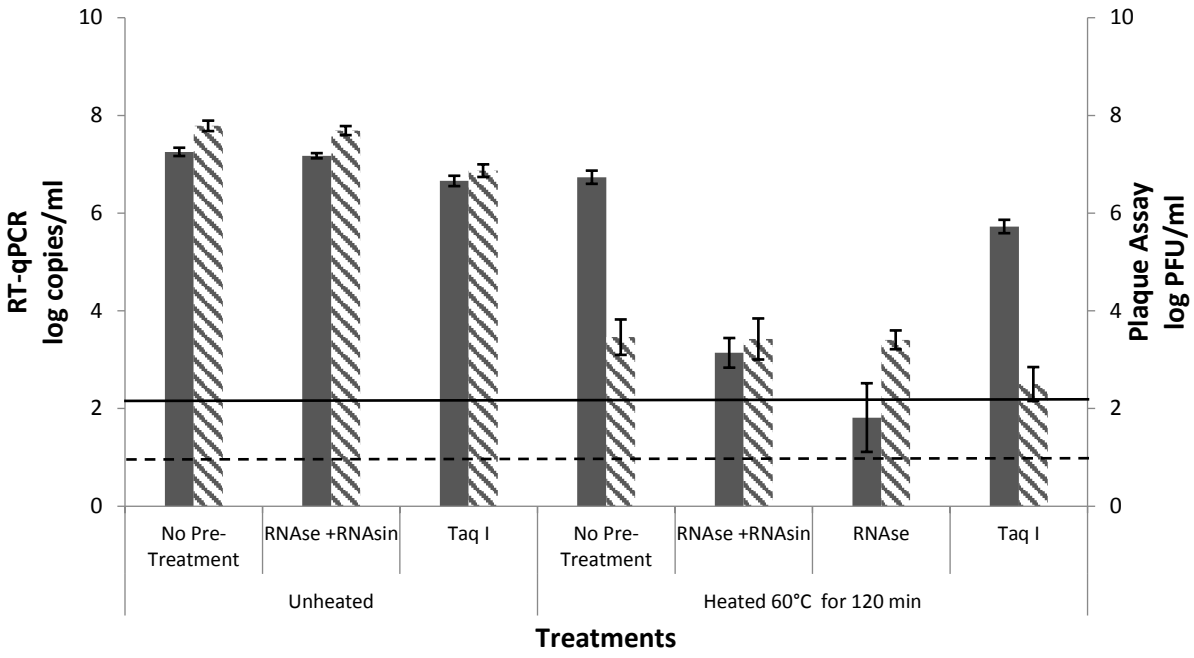


Figure 2-4. Quantification of heat-inactivated MS2 with and without enzyme (RNase+RNasin, RNase or TaqI) pre-treatment analysed by RT-qPCR(■) and plaque assay (▨) with LOQ of RT-qPCR (—) and plaque assay (- -).

Even though the RNase pre-treatment was able to reduce the over-quantification of RT-qPCR, it under-estimated the number of infectious MS2 by 1.5 $\log_{10}$ PFU/ml compared to the plaque assay result. Moreover, TaqI pre-treatment slightly reduced the over-quantification of infectious MS2 by

RT-qPCR assay but it still over-estimated the infectious viral particles by 3 log_{10} PFU/ml. Therefore, RNase alone and TaqI pre-treatment were not applied in the subsequent inactivation studies.

To evaluate whether the enzymatic pre-treatment affects MS2 propagation, the plaque assay results of unheated MS2 with RNase+RNasin and TaqI pre-treatment were compared to unheated MS2 without enzymatic pre-treatment (as a control). The plaque assay result showed no significant difference ($P>0.05$) between the RNase+RNasin pre-treatment and the control (Figure 2-4). In contrast, the plaque assay result of MS2 pre-treated with TaqI showed a significant difference ($P<0.001$) to the control. The TaqI pre-treatment slightly reduced the number of infectious MS2 by 0.92 log_{10} PFU/ml.

2.3.3. The application of RT-qPCR with pre-treatment in inactivation study

In the inactivation study, MS2 was treated with heat or chlorination. Since, from the initial study, exposure to ClO_2 at 0.5 ppm did not inactivate MS2, higher concentrations of ClO_2 were used in the subsequent inactivation study. Heat treatment at 72°C (Figure 2-5) and chlorination with 1 – 16 ppm ClO_2 for 5 min (Figure 2-6) were able to inactivate MS2. The result from plaque assay and RT-qPCR with pre-treatment showed that heating at 72°C for 40 min reduced the number of MS2 up to 5.57 log_{10} PFU/ml and 4.81 log_{10} copies/ml, respectively. Furthermore, the chlorine dioxide treatment for 5 min up to 16 ppm showed the reduction of up to 3.46 log_{10} PFU/ml and 3.46 log_{10} copies/ml, respectively. However, the result of RT-qPCR without pre-treatment showed that both heating at 72°C for up to 40 min and chlorine dioxide treatment for 5 min up to 16 ppm resulted in no MS2 reduction.

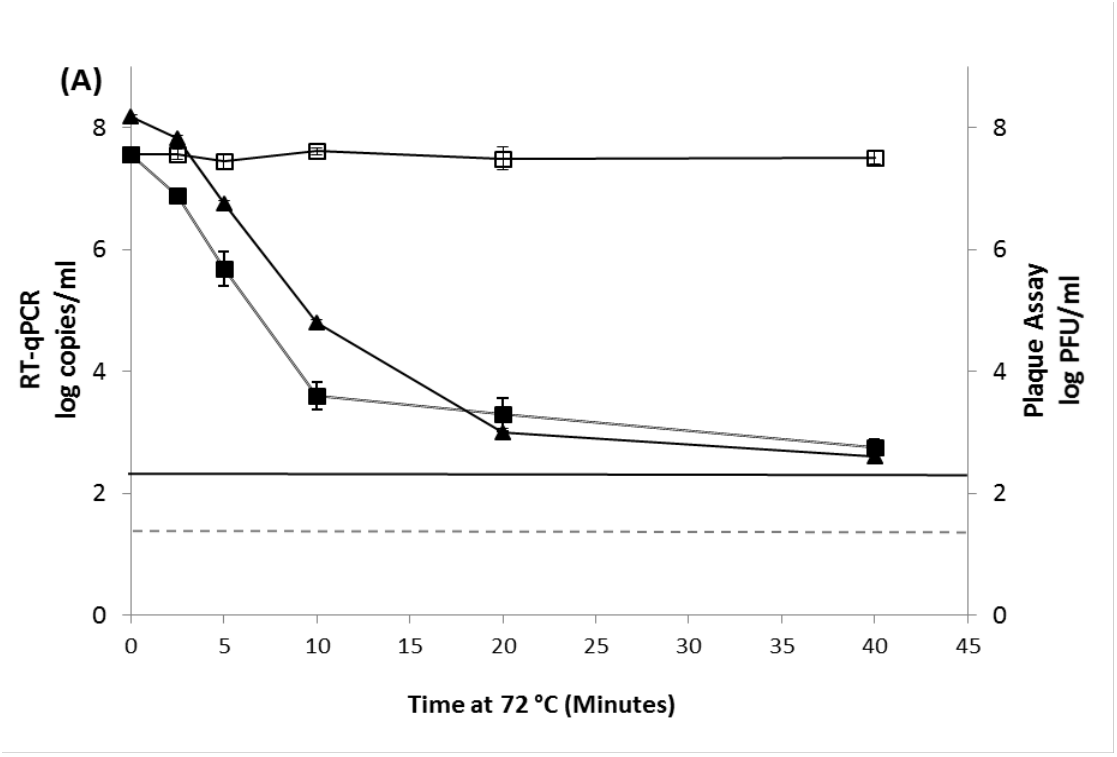


Figure 2-5. MS2 inactivation by heat treatment at 72°C over 40 min as analysed by RT-qPCR without (□) or with RNase+RNasin pre-treatment (■) compared to the plaque assay (▲) with LOQ of RT-qPCR (—) and plaque assay (- -).

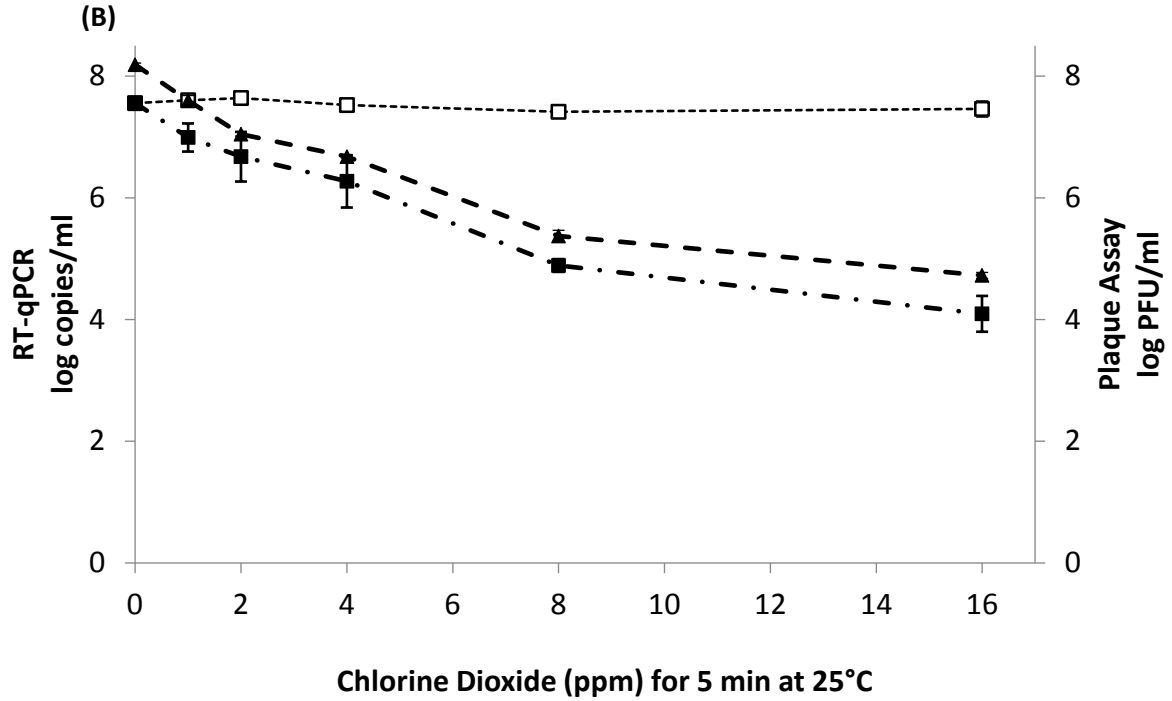


Figure 2-6. MS2 inactivation by exposure to different concentration of chlorine dioxide for 5 min at 25°C, analysed by RT-qPCR without (□) or with RNase+RNasin treatment (■) and plaque assay (▲).

2.4. Discussion

Although human NoV can now be cultured *in vivo* using stem cell-derived human enteroids (Ettayebi *et al.*, 2016) and can be used to qualitatively evaluate the efficacy of disinfectants for NoV inactivation (Costantini *et al.*, 2018), however the development of culture-based assay as a simple, cheap and robust NoV quantification assay remains challenging (Jones *et al.*, 2015). As a solution, the molecular-based methods, such as RT-qPCR have been widely developed and proposed as the detection and quantification assay of NoV (Jones *et al.*, 2015; Kirby & Iturriza-Gómara, 2012; Lowther *et al.*, 2019; Vinjé, 2015). However, the inability of RT-qPCR to distinguish between infectious and non-infectious viral particles is the major limitation of this assay. RT-qPCR without sample pre-treatment may detect and quantify the total nucleic acid from both infectious and non-infectious viral particles, but only infectious NoV particles are able to infect humans and associated with a risk of human illness. Not knowing the real number of infectious viruses in a mixture of infectious and non-infectious virus may lead to overestimation of NoV and lead to inappropriate decisions regarding the risk management of human NoV.

Accordingly, a cultivable NoV surrogate such as MS2 bacteriophage can be used to evaluate the efficacy of RT-qPCR to quantify infectious viral particles by comparing the calculated number of copies of viral particles to the plaque assay in which only infectious viral particles are being quantified. In our studies, the efficacy of RT-qPCR (without pre-treatment) to quantify infectious viral particles from non-inactivated MS2 stock was evaluated by comparing the RT-qPCR to plaque assay results. The RT-qPCR gave comparable results and high correlation (R^2=0.9994 ($P<0.001$) with a slope value of 0.9938 and intercept value of 0.12997) to the plaque assay for the quantification of infectious viral particles (Figure 2-2). The LOQ of RT-qPCR method used in this study was 4.46 copies/reaction or 4.46 copies/25 µl. This result was comparable to RT-qPCR assay from Rolfe *et al.* (2007); Dreier *et al.* (2005); and O'Connell *et al.* (2006) where the LOQ were 2 copies/25 µl, 44.9, and 200 copies/20 µl, respectively.

However, the RT-qPCR failed to quantify the number of infectious MS2 particles surviving high temperature treatment for different durations when compared with the plaque assay results. The numbers of MS2 genomes were constant for all treatments when quantified by RT-qPCR but declined when enumerated by plaque assay (Figure 2-3). This indicates that the quantification of infectious viral particles after heat treatment was over-quantified by RT-qPCR without pre-treatment compared to the plaque assay. In agreement with our result, other studies also reported no correlation between the numbers of genomic copies detected with RT-qPCR (without pre-treatment prior to RNA extraction) and the number of infectious viral particles detected by plaque assay after an inactivation treatment, such as heat (>72°C), chlorination or other type of disinfectant (Baert *et al.*, 2008; Belliot *et al.*, 2008; Escudero-Abarca *et al.*, 2014; Fraisse *et al.*, 2011), but positive correlation on the viral quantification were observed between RT-qPCR with pre-treatments (using RNase or PMA/EMA) and plaque assay results (Escudero-Abarca *et al.*, 2014; Leifels *et al.*, 2015; Parshionikar *et al.*, 2010). However, the efficacy of pre-treatment prior to RNA extraction may vary depends on type of virus, matrix types, inactivation treatments and RNA extraction procedure. For example, pre-treatment using PMA was effective to measure infectious poliovirus surviving from heat treatment, but less effective for NoV (Parshionikar *et al.*, 2010). None of these studies used MS2 as a NoV surrogate, but instead used MNV, FCV, PV or HAV. Therefore, the present results together with those observed in other studies confirm that RT-qPCR without pre-treatment prior to nucleic acid extraction is insufficient to estimate the levels of infectious viral particles, especially when applied to particular inactivation treatments.

In our study, enzymatic pre-treatment prior to nucleic acid extraction was used to eliminate free genomic RNA from the non-infectious MS2 viral particles. The RT-qPCR and plaque assay results of infectious MS2 in the inactivation experiments showed that the over-quantification of infectious MS2 from heat treatment can be reduced with the application of RNase or RNase+RNasin prior to RNA extraction (Figure 2-4). This is because RNase degrades the RNA from non-infectious viral particles that lack capsid protection, so that only RNA from infectious MS2 was quantified by the RT-

qPCR. When MS2 is exposed to 72°C for 10 min, the protein capsid is disrupted (Pecson *et al.*, 2009) and so the RNA genome from the damaged virus becomes accessible to RNase (Brié *et al.*, 2016). The ability of RNase to degrade viral genome integrity also depends on the inactivation method and target viruses used in the assay (Knight *et al.*, 2012; Pecson *et al.*, 2009). For example, in their inactivation study of HAV, Bhattacharya *et al.* (2004) showed that the use of RNase as a pre-treatment in RT-PCR was more effective for UV inactivated samples than when it was used with heat treated samples.

However, RNases may remain active at low temperature and pH, and continue to degrade RNA released from infectious viral particles during the nucleic acid extraction and preparation for PCR assay. For instance, during purification of RNase A from bovine pancreas by a classical procedure, the enzyme remained stable and active under low temperature and pH (Raines, 1998). Therefore, the application of RNase as pre-treatment without further inactivation of this enzyme prior to RNA extraction may result in under-estimation of infectious viral particles. As shown in our enzymatic pre-treatment studies, the application of RNase without further inactivation by RNasin in RT-qPCR assay under-estimated the number of infectious MS2 the heat treatment compared to RNase+RNasin pre-treatment (Figure 2-4).

Furthermore, RT-qPCR with RNase+RNasin pre-treatment also showed similar trend to the plaque assay result. This indicates that RNase+RNasin pre-treatment can be used to reduce the over-estimation of infectious MS2 after exposure to high temperature (Figure 2-5) or chlorine dioxide treatment (Figure 2-6). Our results confirm the observation of Nuanualsuwan and Cliver (2002) that the RNase is able to eliminate the over-estimation of infectious NoV surrogates such as HAV, vaccine PV 1 and FCV from UV, chlorine and 72°C inactivation.

RNasin is a protein that inhibits RNA by binding with high affinity to, and blocking the active site of RNase (Kobe & Deisenhofer, 1996). The addition of RNasin therefore helps to prevent RNA degradation by residual RNase (Nuanualsuwan & Cliver, 2002; Yang & Griffiths, 2014), which might

result in under-estimation of the infectious MS2. In our study, RNasin was used along with the application of RNase as pre-treatment prior to nucleic acid extraction. Results from the RT-qPCR showed that the significant difference ($P<0.001$) between RNase with and without subsequent RNasin treatment was observed (Figure 2-4). Moreover, no significant difference ($P<0.001$) was observed in plaque assay results between RNase+RNasin pre-treatment and no pre-treatment (as a control). These indicated that the enzymatic pre-treatment of RNase followed by RNasin might not injure the infectious MS2 or might not interfere the propagation of infectious MS2 into the host cell. Thus, RNase+RNasin is potentially to be applied as a pre-treatment prior to nucleic acid extraction for the RT-qPCR assay to enumerate the infectious virus from the inactivation.

As an alternative to RNase+RNasin, we evaluated the use of restriction endonucleases such as TaqI as a pre-treatment. This class of enzyme is cheaper than RNase and simpler to use because they provide a one-step pre-treatment rather than the two-step RNase then RNasin protocol. To the best of our knowledge, however, the use of TaqI as a pre-treatment has not been widely reported. Our RT-qPCR results showed that the use of TaqI reduced the over-estimation of MS2 by 1 $\log_{10}$ copies/ml; however, in comparison with the plaque assay, it still overestimated the amount of infectious virus by approximately 3 $\log_{10}$ PFU/ml. In addition, the results of the plaque assay after this pre-treatment indicates that either TaqI may affect the lysogenic cycle of MS2 into the host cell (*E. coli* strain K12) or incubation at 60°C for 30 min may inactivate MS2 as the number of MS2 were approximately 1 $\log_{10}$ PFU/ml lower than in the control (without pre-treatment) (Figure 2-4). As a result, the use of TaqI as a pre-treatment may not be as useful as RNase, and further optimisation is needed before applying this enzyme in future studies.

High temperature has been shown to be an effective treatment to reduce the number of infectious NoV and its surrogates including MS2 either in the foods, shellfish, water or culture medium (Araud *et al.*, 2016; Bozkurt *et al.*, 2014b; Brié *et al.*, 2016; Buckow *et al.*, 2008; Mormann *et al.*, 2010; Tuladhar *et al.*, 2012). It works by changing the structure of the capsid protein of the viruses (Baert *et al.*, 2008; Nuanualsuwan & Cliver, 2003), and potentially jeopardising RNA integrity, which may

affect their ability to initiate the infectious process (Cliver, 2009). We also observed that heat inactivation at 72°C for 20 to 40 min was effective and reduced the number of MS2 by 5-5.5 $\log_{10}$ PFU/ml (Figure 2-5). Moreover, heating at 76.6°C for 2 min has been suggested as the minimum temperature to eliminate 4-5 $\log_{10}$ copies/reaction of NoV by heat inactivation modelling (Beller *et al.*, 1997; Topping *et al.*, 2009). Therefore, the application of heat treatment in food preparation such as steaming, boiling and cooking might be an effective method to eliminate enteric viruses including NoV in food.

Oxidative chemical substances such as chlorine and ClO_2 are alternative disinfectants to inactivate enteric virus on food contact surfaces (Feliciano *et al.*, 2012; Kim *et al.*, 2012) and in uncooked food (Predmore & Li, 2011). Chlorine dioxide causes oxidative damage to the RNA genome and reacts with the capsid protein thus preventing virus attachment to the host cell (Li *et al.*, 2004). A study from Hornstra *et al.* (2011) confirmed that the application of 0.5 ppm ClO_2 using a reactor was sufficient to inactivate MS2 by up to 5 $\log_{10}$ unit after an exposure time of at least 20 min. This contrasts with our preliminary study using the plaque assay that found that the application of 0.5 ppm ClO_2 for 15-60 min did not inactivate MS2. This difference was probably due to the use of a reactor in the previous study which maintains the concentration of ClO_2 constant during the treatment. When higher concentrations of ClO_2, up to 16 ppm for 5 min, were applied in our inactivation studies, ClO_2 inactivated MS2 by up to 3 $\log_{10}$ PFU/ml (Figure 2-6). The different inactivation efficacy between our preliminary and inactivation studies may be due to the tailing phenomenon which occurs during chlorine or ClO_2 inactivation processes (Hornstra *et al.*, 2011; Sigstam *et al.*, 2014); therefore the concentration of ClO_2 is not linearly correlated with the viral inactivation.

Our inactivation studies showed that both heat and ClO_2 treatment have the potential to be applied to eliminate and to reduce viral particles that may contaminate food, water or food contact surfaces. The use of ClO_2 might be a good alternative disinfectant to eliminate or to reduce the viruses that are transmitted to food via the secondary transmission such as contaminated water or infected-

person hand during food handling, but might be ineffective to eliminate NoV inside the shellfish tissue, which originates from the natural contamination. This ClO_2 treatments can be done by dipping, washing or cleaning processes when it is not possible to use heat treatment for uncooked food products such as raw oysters, fresh fruits and vegetables.

2.5. Conclusions

In this study, the quantification of MS2 bacteriophage (as a NoV surrogate) after exposure to heat or chlorine dioxide using RT-qPCR without RNase pre-treatment overestimated the number of infectious viruses, while RT-qPCR with RNase-only pre-treatment underestimated the number of infectious viruses. Hence, the use of RNasin as a complimentary step after RNase pre-treatment is required for the RT-qPCR assay to produce a comparable result to a plaque assay in the quantification of infectious viral particles. The results of the present study, therefore, demonstrate the potential for using such an approach to more accurately determine the infectious viral particles of “uncultivable” virus where the viral capsid integrity is the object of inactivation, such as NoV surviving from inactivation by heat or chlorine dioxide. This pre-treatment might not be suitable to determine surviving viral particles from inactivation by UV or irradiation where the viral genome integrity is the object of inactivation.

Chapter 3. Thermal inactivation kinetics of Human norovirus and MS2 bacteriophage in buffered media and bioaccumulated Tasmanian Blue Mussel (*Mytilus galloprovincialis*)

3.1. Introduction

NoV is one of the most prominent foodborne viruses that cause enteritic disease (Koopmans *et al.*, 2008) and is frequently related to consumption of virus-contaminated shellfish (Le Guyader *et al.*, 2010). There are numerous outbreak reports of NoV contamination from shellfish in U.S.A. (Berg *et al.*, 2000; Kohn *et al.*, 1995), European countries (Le Guyader *et al.*, 2006; Westrell *et al.*, 2010), Australia (Webby *et al.*, 2007), and Singapore (Ng *et al.*, 2005). Although most of the outbreaks caused by NoV were associated with the consumption of raw oysters, undercooked shellfish also contributed to outbreaks (Alfano-Sobsey *et al.*, 2012; Richards, 2006). When cooking is applied, temperature and holding time play important roles during cooking, and are considered as critical points in reducing the incidence of NoV-foodborne cases.

In countries where shellfish is consumed as a cooked meal, the application of thermal inactivation by heating can greatly reduce the risk of gastrointestinal disease, without concerning the change of organoleptic quality. Thermal inactivation is considered as one of the most effective treatments to reduce the number of enteric viruses that contaminated food and drinking water (Bertrand *et al.*, 2012). There is high variability in the efficacy of this treatment, which depends on the matrix types and sizes, the virus species or strains, detection or quantification methods (Bertrand *et al.*, 2012; Bozkurt *et al.*, 2015b) and holding time (Arthur & Gibson, 2015). As expected, inactivation rates at ≥50°C are faster than at <50°C (Bertrand *et al.*, 2012), hence, heating at ≥50°C has potential to be applied in food processing to reduce the risk of NoV infection.

Studies of heat inactivation of enteric viruses have been initiated since 1960's (Heberling & Cheever, 1960). However, determining the heat inactivation kinetics of viruses such as NoV , SaV, and HEV

remains challenging (Bozkurt *et al.*, 2015b; Koopmans & Duizer, 2004; Randazzo *et al.*, 2018) due to the absence of an effective and robust cell culture-based system as a standard quantification method (Harrison & DiCaprio, 2018; Oka *et al.*, 2015). Consequently, molecular-based method such as PCR, and the culturable surrogates that have a similar structure to the targeted viruses, have been commonly applied in heat inactivation studies (Flannery *et al.*, 2014; Randazzo *et al.*, 2018; Richards, 2012). Since the inactivation kinetics of these surrogates is varied, thus, a study comparing the inactivation kinetics between the actual virus and a surrogate would be beneficial to reduce underestimation or overestimation of the inactivation kinetics.

In the last few decades, several mathematical models have been used to describe the viral inactivation kinetics and to evaluate the efficacy of thermal inactivation in reducing enteric viruses in food and water (Deboosere *et al.*, 2004b; Deboosere *et al.*, 2010; Kauppinen & Miettinen, 2017; Romero *et al.*, 2011) or their surrogates (Bozkurt *et al.*, 2013, 2014a; Hewitt *et al.*, 2009). Linear and non-linear regression models have been applied to describe and to predict the inactivation kinetics in these studies. First-order kinetic and log-logistic equations were widely used as linear models to generate D and z values for thermal inactivation of enteric viruses, while Weibull and Biphasic models were used to describe more complex inactivation kinetics (Araud *et al.*, 2016; Bertrand *et al.*, 2012; Seo *et al.*, 2012; Tuladhar *et al.*, 2012). Although some of studies have explored both model types (linear and non-linear) to determine the thermal inactivation kinetics, there are few studies on heat inactivation kinetics of human NoV and its surrogates which incorporate or compare both models.

In this study, a pre-treatment RT-qPCR was used as a quantification method for NoV during the heat inactivation study. Pre-treatment RT-qPCR has been used in some studies to enumerate NoV in the sample that contains both infectious and non-infectious viruses. The use of substances such as EMA, PMA/PMAXX, proteinase K and RNase as a pre-treatment in RT-qPCR has been shown to reduce the overestimation of infectious viral particles (Barbeau *et al.*, 2005; Karim *et al.*, 2015; Nuanualsuwan & Cliver, 2002; Oristo *et al.*, 2018). Also, MS2 bacteriophage has been proposed as a surrogate for NoV

inactivation studies because of its structural similarity with NoV, and because it is easy to handle and cheap (Tufenkji & Emelko, 2011). To the best of the candidate's knowledge, there is no study that has evaluated the heat inactivation kinetics of MS2 and infectious NoV (which was quantified by pre-treatment RT-qPCR) using both linear and non-linear model approaches.

The purposes of the present study were to evaluate and to compare thermal inactivation kinetics of NoV and its surrogate (MS2) in buffered media and Tasmanian Blue Mussel *(Mytilus galloprovincialis*) matrix utilising different models (*i.e.* the log linear, Weibull and Biphasic model). Mussels were artificially contaminated by the bioaccumulation process to mimic the actual condition of enteric virus's transmission routes in shellfish. Viruses in buffered media and contaminated-mussel were treated with different temperatures and holding times.

3.2. Materials and methods

3.2.1. NoV stock preparation

Eight fresh faecal specimens containing NoV genogrup II genotype 4 (GII.4) were provided by the Hobart Pathology, Hobart, Tasmania. These samples were previously determined to be NoV-positive by an immunochromatographic test using Rida®Quick (Biopharm AG, Gemany) (Bruggink *et al.*, 2011; Bruins *et al.*, 2010; Kirby *et al.*, 2010). All samples were prepared as previously described by Trujillo *et al.* (2006) with some modifications, described here. In brief, 1 g of faecal/stool or 1 ml of watery stool was suspended in 9 ml of PBS (Phosphate Buffered Saline) that was previously prepared in diethyl pyrocarbonate-treated water, yielding a 10% suspension. The suspension was then added to 5 ml chloroform and vigorously shaken for 30 sec. The virus was then separated from the organic matter by centrifugation at 10,000 x g for 10 min at 4°C. The upper aqueous phase was transferred to new, sterile, 50 ml plastic tubes and serially filtered through 0.45 and 0.22 µm pore-size low-protein-binding membrane filters (Millipore, USA). The virus stock was stored at -80°C for subsequent studies. RT-qPCR assay with enzyme pre-treatment was performed to determine the NoV concentration on the virus stock. The specific primers COG2R and QINF2 were used to quantify

the NoV GII.4 because of their specificity and sensitivity (International Organization for Standardization, 2013; Loisy *et al.*, 2005; Miura *et al.*, 2013). Virus stocks with concentration of >10^9 genomic copies per ml were used for inactivation studies.

3.2.2. MS2 bacteriophage stock production

MS2 bacteriophage (MS2) was produced as previously described in Section 2.2.1. of this thesis. The concentration of infectious MS2 in the stock was confirmed by a double layer agar method (EPA, 2001). The concentration of infectious MS2 was expected to be between 10^{11} to 10^{12} PFU/ml.

3.2.3. Bioaccumulation in mussels

Five kilograms of live Tasmanian Blue Mussel *(Mytilus galloprovincialis)* were purchased from a single local supplier in Tasmania and kept at <10°C during transportation. Three individual mussels per batch (1 batch equal to 1 kg mussel) were randomly picked and analysed by RT-qPCR and plaque assay to detect the presence of MS2 and NoV as natural contaminants. In the screening step, naturally contaminated batches of mussels (with MS2 or NoV) and mussels with broken shells were not used for the bioaccumulation study. None of the mussel batches were naturally contaminated by NoV and MS2.

Only four kilograms of mussels (50-60 individual mussels/kg/batches) were obtained from the screening step, and then were acclimated for 24 h in an aquarium (40 x 25 x 50 cm) using 20 l of sterile artificial seawater with continuous aeration. After the acclimatisation, 100 live mussels were selected for the bioaccumulation process. The mussels were laid on a monolayer disposal in 10 l of sterile artificial sea water (containing 2% of sea salt) which was contaminated with NoV and MS2 stock. The final concentration of NoV and MS2 in the aquarium seawater was approximately 10^7-10^8 copies/ml and 10^8-10^9 PFU/ml, respectively. To optimize the bioaccumulation process in the DT of mussels, 10 ml of concentrated phytoplankton (Reef Phytoplankton™, Australia) was added to the seawater. The bioaccumulation process was conducted for 12 and 24 h at 10 ± 4°C, under similar condition to the acclimatisation step. After bioaccumulation process, all mussels were dipped in 20 l

sterile seawater for 5 min to remove contaminated-water from the mussel body. Three individual mussels were dissected to take out the tissue. The mussel tissues were washed with sterile saline water (ddH_2O+0.9% NaCl), extracted and then analysed using RT-qPCR and/or plaque assay to quantify the NoV and MS2 concentration. Each mussel tissue was weighed and recorded prior to sample extraction. The bioaccumulation process is presented in Figure 3-1.

3.2.4. Thermal inactivation in buffered media

The temperature of buffered media (PBS) in 15 ml plastic tubes was equilibrated by heat pre-treatment for 10 min at 60, 72 and 90°C for thermal treatments, or at 20°C for controls. NoV and MS2 stocks were added to make final concentrations of approximately 10^7 copies/ml and 10^8 PFU/ml, respectively. The suspensions were heated using water bath at 60±1°C for 15, 30, 60, 120 and 240 min; 72±1°C for 2.5, 5, 10, 20 and 40 min; and 90±1°C for 1, 2.5, 5, 10, and 20 min. Each treatment was done in triplicates. Thermocouple Tecpel 319® (Taiwan) with 4-channel wired probes were used to confirm the actual temperature in the tubes during treatment. After each incubation time, samples were taken from water bath and kept in a freezer at -20°C for further analysis.

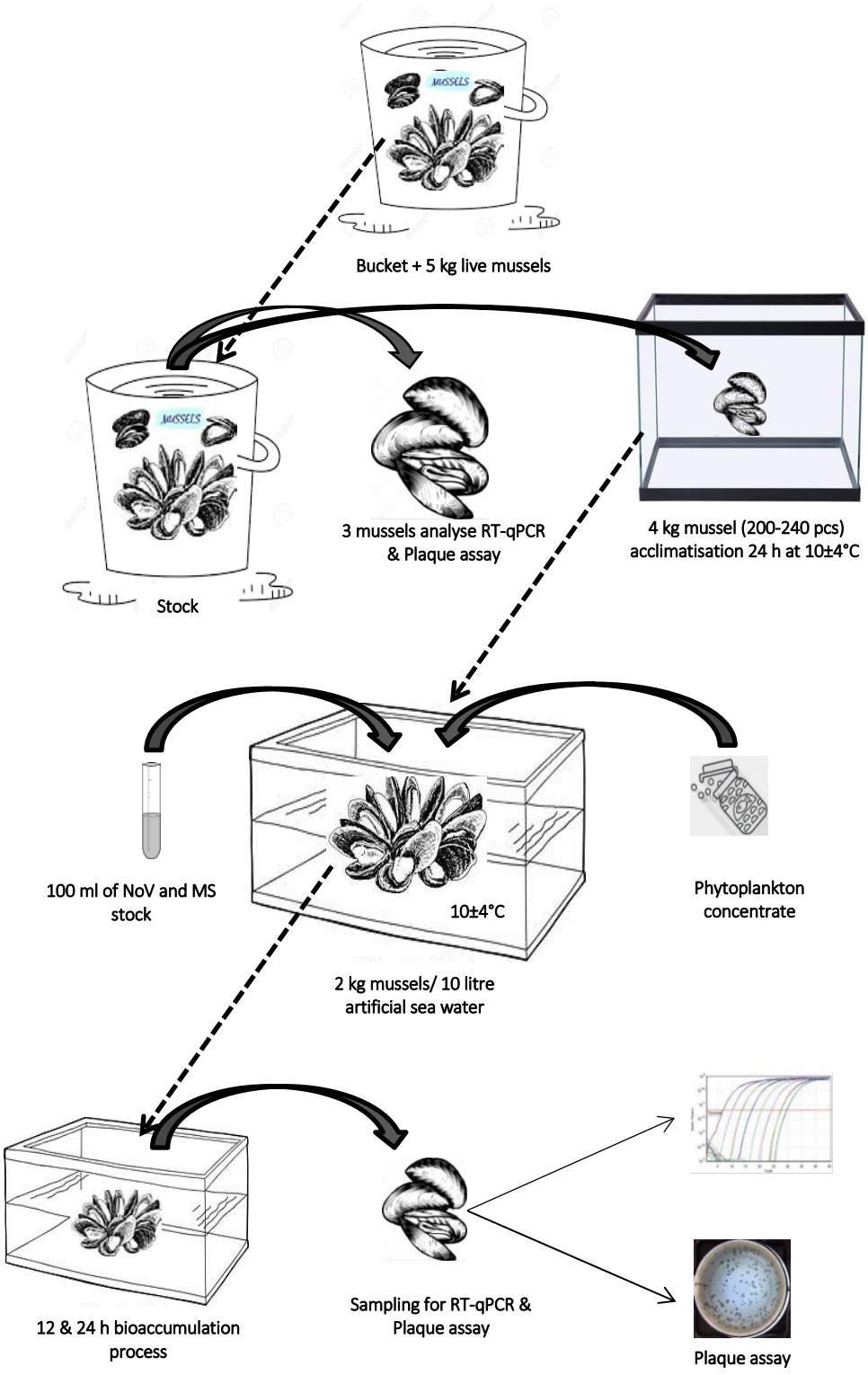


Figure 3-1. Acclimatisation and bioaccumulation process of Tasmanian Blue Mussel *(Mytilus galloprovincialis)*

3.2.5. Thermal inactivation in mussel matrix

The thermal inactivation treatments were done in triplicate in water baths at 60, 72 and 90°±1C. Previously, forty five of 30 ml of PBS solution in 50 ml plastic tubes were pre-heated at certain temperatures (for 30 min) to equilibrate the thermal condition. The PBS solution was used as a buffer media in this study to avoid a viral aggregation due to changes in the environment before the heat treatment. Two pieces of bioaccumulated-mussels tissue (approximately 10-14 g) were then added to each suspension/tube and heated for specified contact times as shown in Table 3-1. After each incubation period, five grams of mussels were removed from the water bath and transferred to a freezer at -20°C before subsequent concentration and purification steps.

Table 3-1. Contact times of thermal inactivation at different temperatures.

Treatments	Replication	Contact Time (Min)
Control (No Heating/±20°C)	3	0
60°C	3	15; 30; 60; 120; and 240
72°C	3	2.5; 5; 10; 20; and 40
90°C	3	1; 2.5; 5; 10; and 20

3.2.6. Enumeration of NoV and MS2

3.2.6.1. Virus concentration

Viruses were isolated and concentrated form the mussel samples following the procedure of Lewis and Metcalf (1988) and Mullendore *et al.* (2001), with modifications. In brief, 5 g of whole mussel tissue were homogenized by shaking at 250 rpm with 1:6 (w/v) 10% tryptose phosphate broth (TPB) in 0.05 M glycine (pH 9.0) for 30 min at 4°C. Seven ml of supernatant was transferred into new 15 ml plastic tubes, 5 ml of chloroform was added, and centrifuged at 10,000 x *g* for 10 min at 4°C. The upper aqueous phase was transferred into 7 ml of 16% PEG 6000 (Sigma Aldrich, USA) and 0.6 M NaCl (pH 6.5), and was shaken at 250 rpm for 12 h at 4°C. The resulting suspension was centrifuged at 10,000 x *g* for 30 min at 4°C. The PEG-containing supernatant was discarded, and the pellet was

suspended in 1 ml PBS, pH 7.5 sonicated for 30 s, shaken for 20 min at 250 rpm. The suspension was re-purified by adding an equal volume of chloroform, and centrifuged at 10,000 x *g* for 10 min at 4°C. The upper aqueous phase was then transferred into new 2 ml micro tube and stored at -20°C.

3.2.6.2. Enzymatic pre-treatment prior to RNA extraction

Prior to nucleic acid extraction, heat treated, as well as control samples, were enzymatically pre-treated as previously described in Section 2.2.4. of this thesis. Subsequently, RNA in samples was extracted by the acid-guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (2006), with modification, as previously described in Section 2.2.2.2.b. of this thesis.

3.2.6.3. Quantification of infectious NoV by RT-qPCR assay

For NoV GII assay, the RT-qPCR was performed as previously described by Jothikumar *et al.* (2005) with modifications using PowerSYBR® Green RNA-to-CT™1-Step Kit (Applied Biosystem, USA) on the Rotor-Gene Q (Qiagen, Germany). JJV2F and COG2R primers were used as forward and reverse primers, respectively. In the final mixture, the RT-qPCR reaction contained 5 µl of RNA template, 0.5 µl of each primer (final concentration of 250 nM), 10 µl of 2x PowerSYBR® Green buffer, 0.2 µl RT-Taq enzyme, and DNase/RNase-free purified-water to make a final volume of 20 µl. The mixture was then subjected to a one-step assay by using the following amplification conditions: (i) RT for 30 min at 48°C, (ii) 10 min at 95°C to activate Taq polymerase, and (iii) 45 cycles of 10 s at 94°C, 20 s at 55°C, and 15 s at 72°C. To develop a standard for enumeration of NoV GII, a plasmid was constructed by cloning nucleotides from 4830-5285 of GII.4 Lordsdale NoV sequences (GenBank accession no. X86557) from isolated NoV. The 475 bps plasmid fragment encompassed 97 bps of RT-qPCR product sequences. The plasmid was purified and serially diluted in free DNase/RNase purified-water. To assess the specificity of PCR products, negative controls using RNA from *E. coli* K12 bacteria and MS2 was used, and the melt curve analysis were performed following the procedures from the Rotor-Gene Q® (Qiagen, Germany).

3.2.6.4. Quantification of infectious MS2 by plaque assay

The infectious MS2 was quantified by plaque assay as described in Section 2.2.2.2.a of this thesis.

3.2.7. Modelling of thermal inactivation kinetics

Three different models *i.e.* log linear (first-order kinetic), Weibull and Biphasic, were compared to obtain the best fitted survival curve of NoV and MS2. The first-order kinetic model is written as follows (Geeraerd *et al.*, 2000):

$$log\left(\frac{N}{N_0}\right) = -\frac{t}{D} \quad \text{or} \quad log10(N) = log10\,(N_0) - \frac{kmax.t}{ln(10)} \qquad \text{(Equation 3-1)}$$

In the cases of shoulder and/or tailing phenomenon, the modified log-linear model with shoulder and/or tailing can be applied to fit the curves. The modified model proposed by Geeraerd *et al.* (2000) is written as follows.

$$\frac{dN}{dt} = -kmax.N.\left(\frac{1}{1+C_c}\right).\left(1 - \frac{N_{res}}{N}\right) \qquad \text{(Equation 3-2)}$$

where *N* is the number of viruses survived after heat treatment and N_o is the initial population of viruses. MS2 population was described in PFU/ml, while NoV was quantified in copies/ml. *t* is the exposure time (min), *kmax* is the first order inactivation constant [1/min] and D (decimal reduction time) is the time required to eliminate 90% of the population (min). Herein, C_c is related to the physiological state of cells or viruses [-], and N_{res} is the residual population density (PFU/ml or copies/ml).

The Weibull model equation proposed by Mafart *et al.* (2002) is described as follows:

$$log\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^p \quad or \quad n = \left(\frac{t}{\delta}\right)^p \qquad \text{(Equation 3-3)}$$

The modified Weibull model which describes concave, convex or linear curves followed by tailing can be used to fit data with tailing phenomenon. The model was proposed by Albert and Mafart (2005) and can be written as follows:

$$log10(N) = Log10\left[(N_0 - N_{res}).10^{\left(-\left(\frac{t}{\delta}\right)^p\right)} + N_{res}\right] \qquad \text{(Equation 3-4)}$$

where δ is the time to first decimal reduction, *p* is a shape parameter, and *n* represents the decimal reduction ratio. The value of *δ* is not equal to the conventional D value. Therefore, *n* can be used to calculate $\log_{10}$ reductions (D), from which 1D can be calculated as *n*=1, or 2D equal to *n*=2.

The biphasic model equation (Geeraerd *et al.*, 2005; Schielke *et al.*, 2011) can be generated from Cerf (1977), as described below:

$$log(N) = log(N_0) + log\left(f.e^{-kmax_1.t} + (1-f).e^{-kmax_2.t}\right) \qquad \text{(Equation 3-5)}$$

where *f* is the fraction of initial population in a major subpopulation, $kmax_1$ and $kmax_2$ is specific inactivation rate (1/time unit) at phase 1 (Initial) and 2 (Tailing), respectively.

Curves were fitted to those three models using GInaFiT for Microsoft Excel (Geeraerd *et al.*, 2005). The 2D, 4D and D Initial values were calculated using Solver® Add-in of Microsoft 365 (Microsoft Corp).

3.2.8. Statistical analysis

The models were evaluated for the best fit by comparing the Root Mean Square Error (RMSE) and the coefficient of determination (R^2) value for the various models. To measure goodness-of-fit, the RMSE was used for both linear and non-linear models (Ratkowsky, 2004), while the R^2 was only used for linear models. The RMSE and R^2 values were calculated using Microsoft Excel® software. The RMSE and R^2 equation are described below:

$$RMSE = \sqrt{\frac{\Sigma(predicted-observed)^2}{n-p}}$$ (Equation 3-6)

$$R^2 = \frac{n(\sum xy)-(\sum x)(\sum y)}{\sqrt{[n\sum x^2-(\sum x)^2][n\sum y^2-(\sum y)^2]}}$$ (Equation 3-7)

where *n* is the number of observations and *p* is the number of parameters to be estimated. The value of *x* presents the independent variables (temperature), while *y* presents the dependent variables (observed values).

3.3. Results

3.3.1. Bioaccumulation of NoV and MS2 in mussel

The preliminary screening confirmed that there were undetectable levels of NoV or MS2 in batches of mussel used in the bioaccumulation study. Four batches of mussels (Approximately 50-60 mussels/batch) were acclimated for 24 h. During 12 and 24 h of bioaccumulation process, the mussel was contaminated by NoV at 6.64 and 6.61 $\log_{10}$ copies/g and MS2 at 7.80 and 7.57 $\log_{10}$ PFU/g, respectively. There were no significant differences ($p>0.05$) in viral concentration in mussels that were subjected to bioaccumulation for 12 or 24 h for either virus as shown in Table 3-2. However, unopened shells were observed in the mussels (<10%) after 24 h bioaccumulation process which may indicate dead or inactive mussels. Therefore, only mussels from the 12 h of bioaccumulation were used for inactivation studies to reduce the variability of initial concentration of NoV or MS2.

Table 3-2. The concentration of NoV and MS2 in seawater and mussel after bioaccumulation process for 12 and 24 h.

Virus	Seawater (24 h)	Unit	Mussel		Unit
			12 h	24 h	
NoV	7.67 ± 0.05	$\log_{10}$ copies/ml	6.64 ± 0.17[a]	6.06 ± 0.63[a]	$\log_{10}$ copies/g
MS2	8.12 ± 0.24	$\log_{10}$ PFU/ml	7.80 ± 0.03[b]	7.57 ± 0.27[b]	$\log_{10}$ PFU/g

**The same letter in the same row denotes no significant differences (p>0.05)*

3.3.2. Thermal inactivation of NoV and MS2

To determine the thermal inactivation kinetics of NoV and MS2 in buffered media and mussel matrix, the virus stock and bioaccumulated-mussels were exposed to heat treatment at 60, 72 and 90±1°C for defined periods. The concentration of infectious NoV and MS2 after heating in both matrices, expressed as log_{10} copies/g or copies/ml and log_{10} PFU/g or PFU/ml, were plotted against the contact time (min) at each temperature as shown in Figure 3-2 to 3-5. The average initial concentrations of NoV in buffer and mussels were 6.26 ± 0.16 log_{10} copies/ml and 6.64 ± 0.17 log_{10} copies/g, respectively. While the MS2 initial concentrations were 7.89 ± 0.07 log_{10} PFU/ml in buffer and 7.80 ± 0.03 log_{10} PFU/g in mussel matrix.

The average of NoV reduction in buffered media by heating at 60°C for 240 min, 72°C for 40 min and 90°C for 20 min were 2.81, 2.96 and 3.88 log_{10} reductions respectively, while the inactivation in mussel matrix were 2.85, 3.08 and 3.58 log_{10} reductions at the end of treatment. Furthermore, the inactivation of MS2 at 60, 72 and 90°C in buffered media and mussel matrix resulted in 4.93, 6.73 and 7.09 and 4.64, 5.42 and 6.35 log_{10} reductions, respectively. Apparently, based on the log reductions trends after the treatment, MS2 were more susceptible to heat treatment than NoV in both buffered media and mussel matrix at each heating temperature. For example, the average of MS2 reduction in buffered media by heating at 72°C for 10 min resulted in 4.74 log_{10} reductions, two logs higher than the reductions of NoV from similar treatment, which was only 2.03 log_{10} reduction. Moreover, similar treatment in the mussel matrix reduced MS2 by 3.30 log_{10} reductions in average, while only 2.35 log_{10} reductions were observed from NoV.

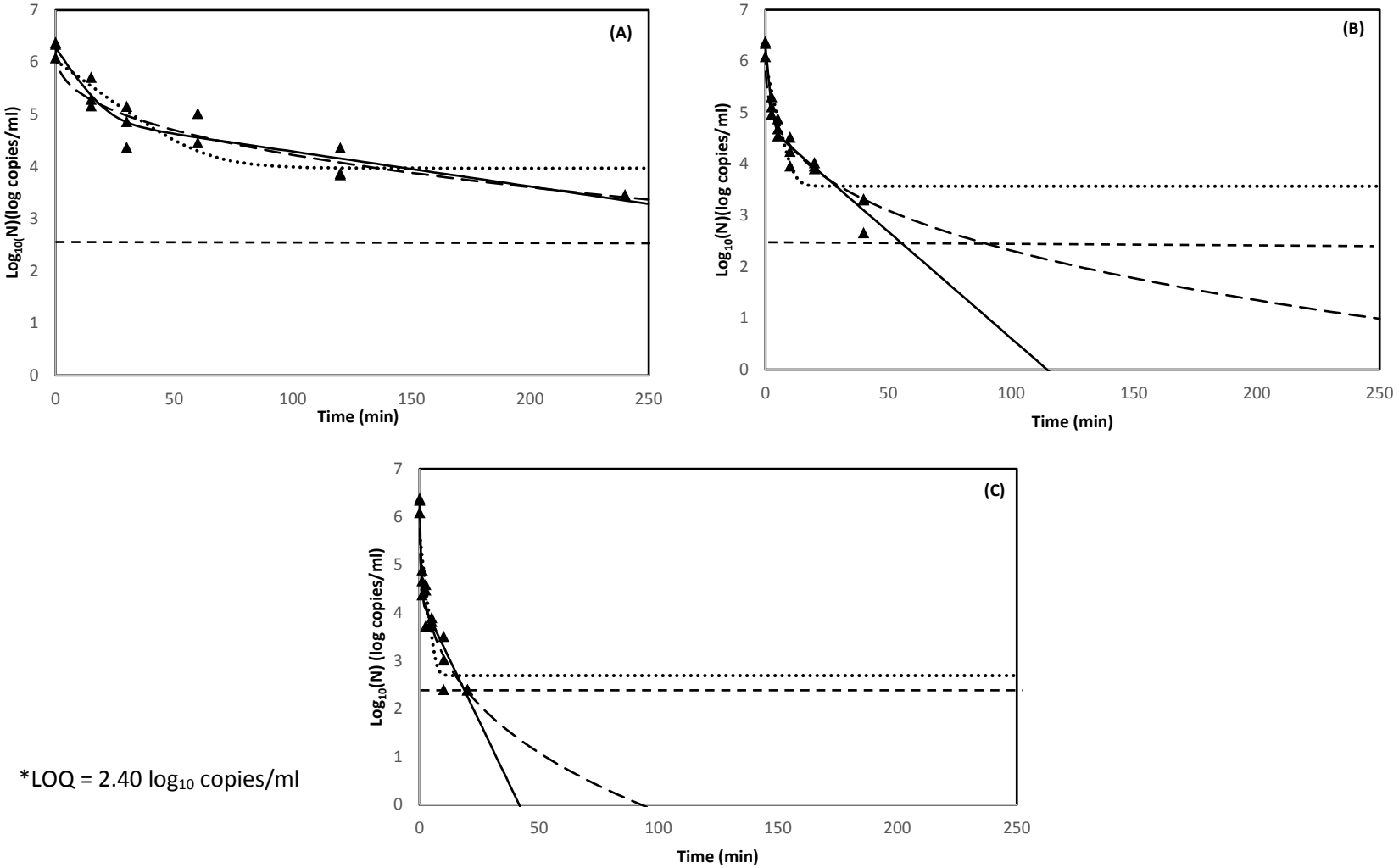
3.3.3. Model fitting and comparison

Linear and non-linear models (see Section 3.2.7.) were used to describe the inactivation kinetics and the times required to a log_{10} reduction (D value) of NoV and MS2 due to thermal inactivation over the time. The data of infectious NoV and MS2 over time during heat treatments at 60, 72 and 90±1°C were fitted using log linear, Weibull and Biphasic models. Since the observed survival of NoV and

MS2 data showed a tailing and/or shoulder phenomenon (Figure 3-2 to 3-5), the models were calculated by modified equations that included terms for tailing and/or shoulders (see Section 3.2.8). RMSE and/or R^2 were used to compare linear and non-linear models, and were also used to determine the best predicted 2D (time to 100-fold reduction) and 4D (time to 10,000 fold reduction) values. During the model fitting, the unmodified log linear model produced a lower coefficient of determination (R^2) compared to the log linear model with tailing and/or shoulder (data not shown), therefore only the modified (with tailing and/or shoulder) log linear model was used for model comparison.

In general, Weibull (without tailing) and Biphasic models presented better predictions of thermal inactivation kinetics in both matrices for both viruses, as shown on Table 3-3 and 3-4. Some inactivation curves were better fitted by Weibull-tailing or Log linear-tailing model than Weibull or Biphasic, especially to predict the infectious viruses in the full duration of the treatment. However, with the assumption that viruses will not survive from heating for extended periods, Weibull-tailing and Log linear-tailing models failed to predict the infectious viruses for extended periods (outside of the full duration of the treatment), because of a constant survival of viruses after certain exposure time (Figure 3-2 to 3-5). Moreover, based on these observations, Weibull (without tailing) was better to predict the infectious viruses from heat treatment in the buffered media, while Biphasic performed better to predict the virus survival in the mussel matrix.

By visually comparing the observed data to the fitted curves of each model, the log linear with tailing model underestimated and/or overestimated the observed infectious population of NoV and MS2, especially at initial contact time (t=0) and the end of treatment, while the Weibull or Biphasic models presented better predictions (Figure 3-2 to 3-5). These subjective evaluations were consistent with curve fitting analyses (Table 3-3 and 3-4) where the RMSE value of the log linear-tailing model were always higher than the Weibull or Biphasic, except for NoV heated at 60°C in mussel matrix.



*LOQ = 2.40 log_{10} copies/ml

Figure 3-2. Thermal inactivation curves of NoV at 60 (A); 72 (B) and 90°C (C) in buffered media fitted with Log linear-tailing (⋯), Weibull (– – –) and Biphasic (—) model.

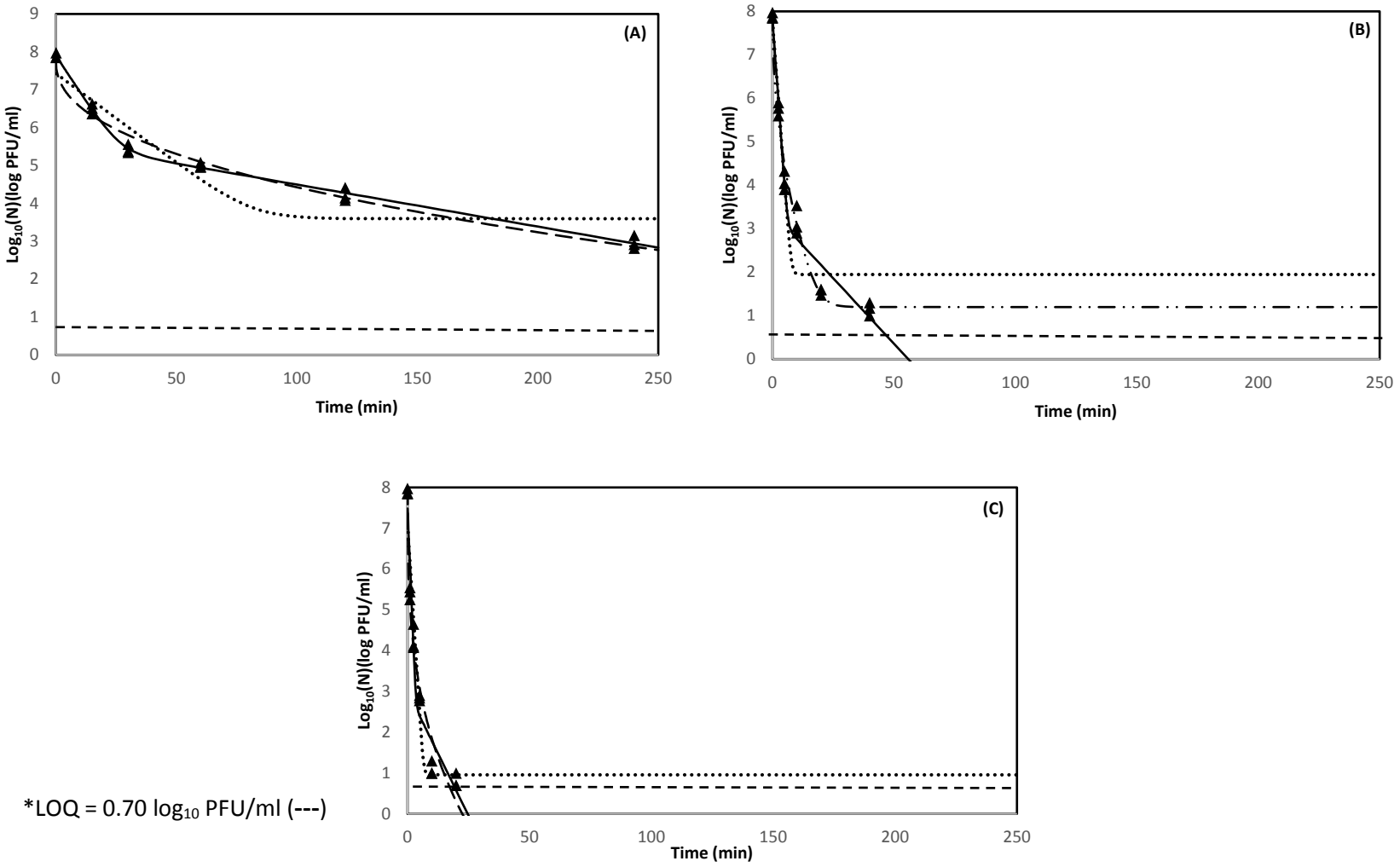
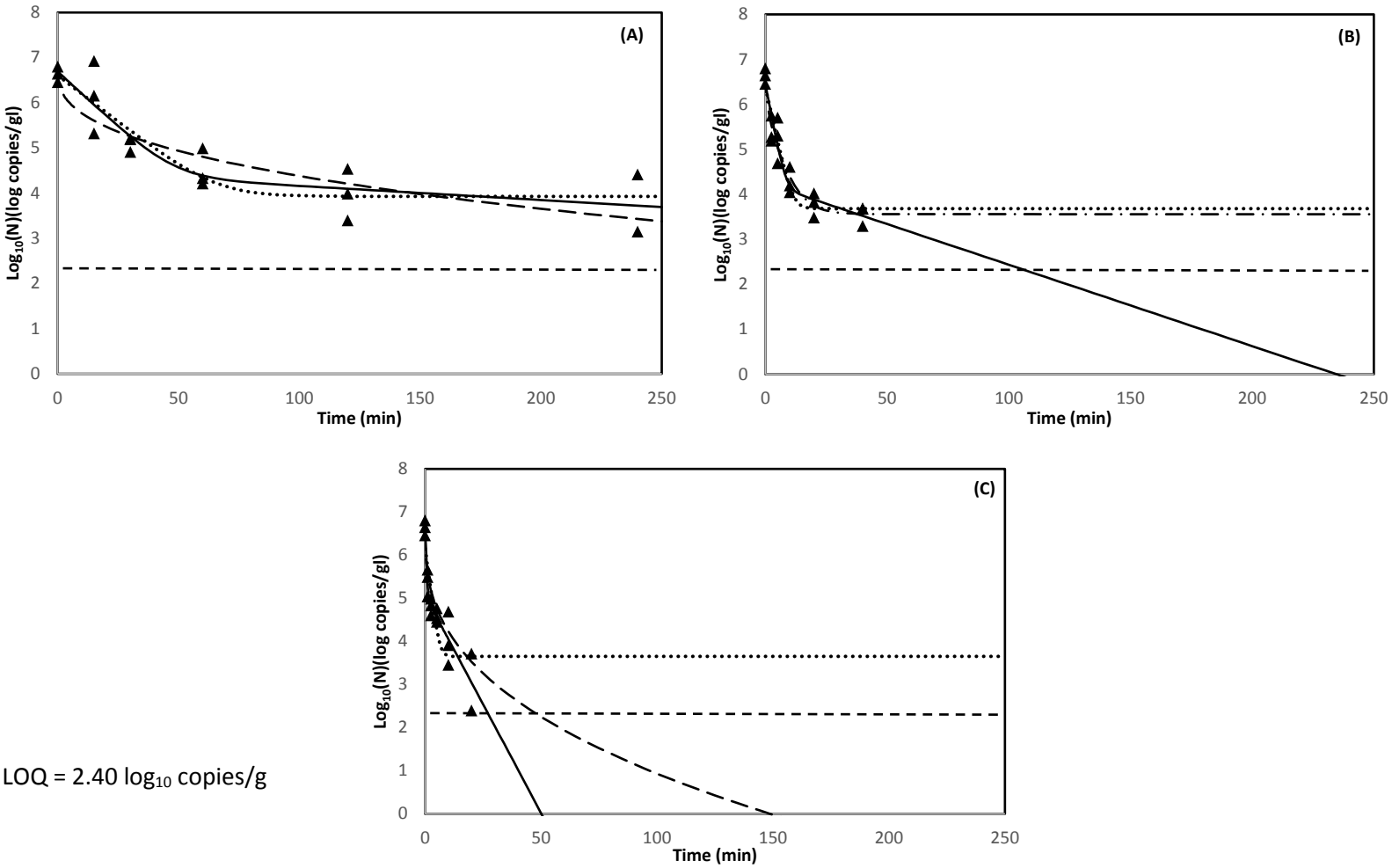
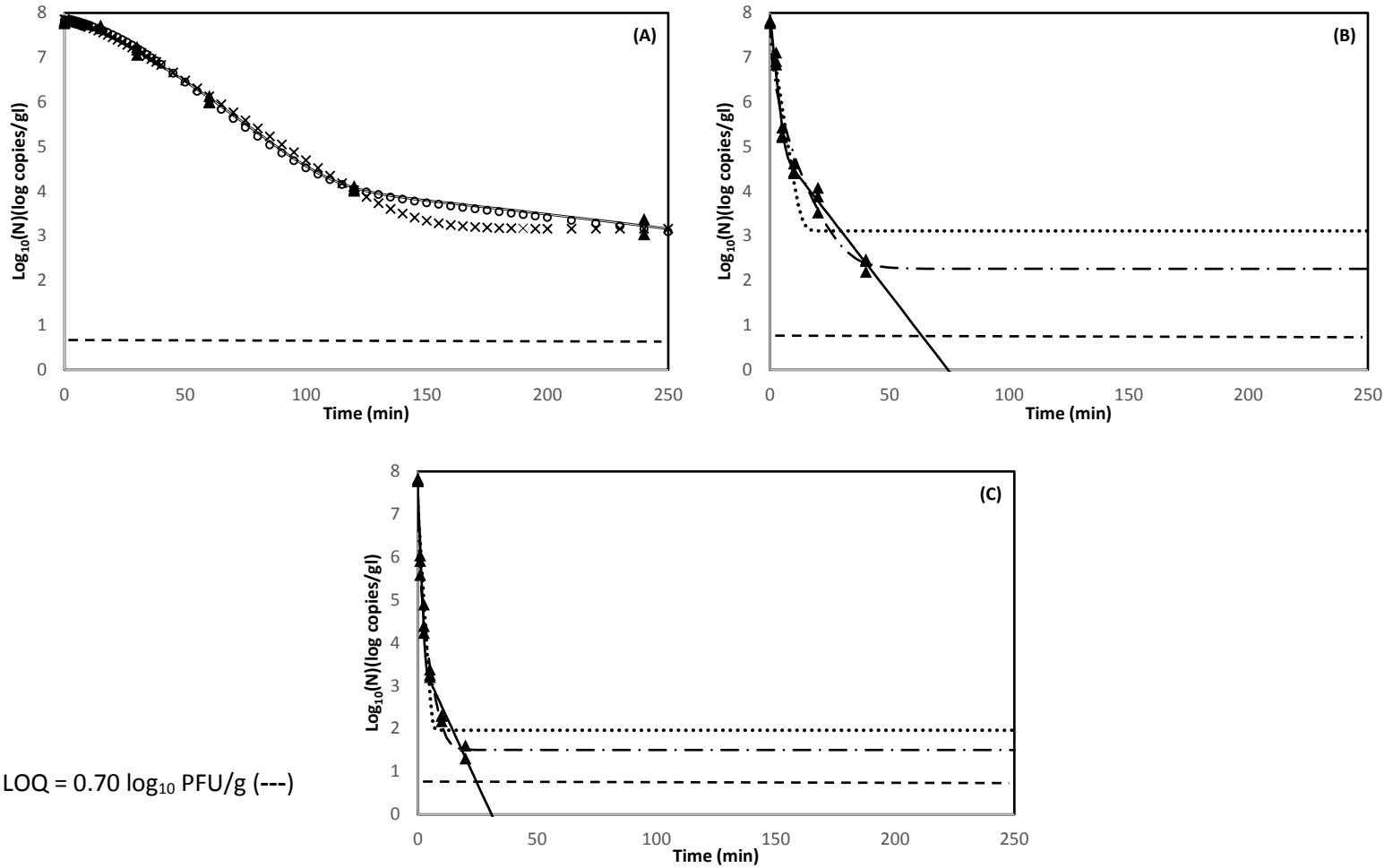


Figure 3-3. Thermal inactivation curves of MS2 at 60 (A); 72 (B) and 90°C (C) in buffered media fitted with Log linear-tailing (⋯), Weibull (– – –), Weibull-tailing (– · –) and Biphasic (—) model.



*LOQ = 2.40 log_{10} copies/g

Figure 3-4. Thermal inactivation curves of NoV at 60 (A); 72 (B) and 90°C (C) in mussel matrix fitted with Log linear-tailing (⋯), Weibull (– – –) , Weibull-tailing (– · –) and Biphasic (—) model.



*LOQ = 0.70 $\log_{10}$ PFU/g (---)

Figure 3-5. Thermal inactivation curves of MS2 at 60 (A); 72 (B) and 90°C (C) in mussel matrix fitted with Log linear-tailing (⋯), Log linear-shoulder-tailing (xxx), Weibull-tailing (– · –), Two-mixed Weibull (═), Biphasic (—) and Biphasic-shoulder (ooo) model

3.3.4. The z curves of NoV and MS2 thermal inactivation.

The calculated D, 2D and 4D values from each thermal inactivation model of NoV and MS2 in buffered media and mussel matrix are presented in Table 3-3 and 3-4. The calculated D values from the best fitted of three models (which has the lowest RMSE for non-linear models or the closest coefficient of determination (R^2) to 1 for linear models) (Table 3-3 and 3-4) were plotted against the temperature of the treatment to generate a general secondary model (z curves) of thermal inactivation (Figure 3-6). For comparison to the general z curves, specific secondary models of Biphasic (Figure 3-7 and 3-8) were derived from D values of Biphasic model only.

In general, the modified Log linear and Weibull with tailing models were failed to calculate the 4D values from thermal inactivation of NoV but were successful for MS2. As expected in this study, the fastest time to reduce 4 log concentrations (4D value) of the viruses in buffered media and mussel matrix were observed from heating at 90°C for less than 1 min.

Table 3-3. The predicted time to log reduction at D, 2D and 4D and the calculated RMSE values from the thermal inactivation curves of NoV in different matrices fitted by Log Linear, Weibull and Biphasic models.

Initial Conc.	Matrix	Temp. (°C)	Model fitting													
			Log Linear					Weibull					Biphasic				
			Time to log reduction (mins)			RMSE	Curves	Time to log reduction (mins)			RMSE	Curves	Time to log reduction (mins)			RMSE	Curves
			D	2D	4D			D (*n*=1)	2D	4D			$D(D_{initial})$	2D	4D		
6.27 ± 0.16 log copies/ml	Buffered Media	60	30.83	84.29	n/a	0.405	Tailing	**15.04**	93.04	575.80	0.296	Normal	16.75	101.43	399.85	0.301	Normal
		72	4.71	10.32	n/a	0.415	Tailing	**1.28**	11.45	102.98	0.172	Normal	2.25	12.05	60.21	0.218	Normal
		90	2.29	4.68	n/a	0.541	Tailing	**0.24**	2.27	21.54	0.303	Normal	0.57	1.50	19.91	0.352	Normal
6.64 ± 0.17 log copies/g	Mussel	60	**24.74**	51.46	n/a	0.511	Tailing	11.09	66.14	393.11	0.587	Normal	20.68	45.39	573.05	0.519	Normal
		72	4.27	8.88	n/a	0.359	Tailing	**2.06**	7.89	n/a	0.313	Tailing	3.66	8.10	99.73	0.356	Normal
		90	2.58	5.50	n/a	0.590	Tailing	1.21	7.18	42.58	0.485	Normal	**0.77**	4.19	24.01	0.397	Normal

Note: The D value predicted from the best fitted models (with the lowest RMSE value) were written in bold and used to create z curves.

Table 3-4. The predicted time to log reduction at D, 2D and 4D and the calculated RMSE values from the thermal inactivation curves of MS2 in different matrices fitted by Log Linear, Weibull and Biphasic models.

Initial Conc.	Matrix	Temp. (°C)	Model fitting													
			Log Linear					Weibull					Biphasic				
			Time to log reduction (mins)			RMSE	Curves	Time to log reduction (mins)			RMSE	Curves	Time to log reduction (mins)			RMSE	Curves
			D	2D	4D			D (*n*=1)	2D	4D			$D(D_{initial})$	2D	4D		
7.89 ± 0.07 log PFU/ml	Buffered Media	60	21.13	42.43	n/a	0.564	Tailing	6.41	31.06	150.34	0.237	Normal	**10.43**	22.04	153.69	0.127	Normal
		72	1.35	2.63	5.39	0.692	Tailing	**0.39**	1.61	6.78	0.290	Tailing	1.29	2.58	5.27	0.394	Normal
		90	1.08	2.16	4.32	0.593	Tailing	**0.04**	0.31	2.67	0.482	Normal	0.36	1.43	2.91	0.574	Normal
7.80 ± 0.03 log PFU/g	Mussel	60	39.61	67.93	125.89	0.114	Shoulder-tailing	39.01	66.32	142.13	0.117	Double-Weibull	**40.40**	65.16	139.70	0.102	Shoulder
		72	2.92	5.82	12.09	0.579	Tailing	1.05	4.25	17.23	0.421	Tailing	**1.97**	3.99	17.80	0.228	Normal
		90	1.15	2.30	4.64	0.549	Tailing	**0.16**	0.82	4.11	0.245	Tailing	0.76	1.52	3.33	0.338	Normal

Note: The D value predicted from the best fitted models (with the lowest RMSE value) were written in bold and used to create z curves.

The general z curves (Figure 3-6) showed that temperature and matrix type affected the D values of NoV and MS2. The intercept values of the curves (calculated from the log linear regression curves) in mussel matrix was always higher than in buffered media for both NoV and MS2 (data not shown). In addition, the predicted D values in buffered medium were constantly lower than in mussel matrix at temperature more than 50°C by a constant proportion (Figure 3-6). The D values of NoV were generally higher than MS2 in both buffered medium and mussel matrix, for each temperature studied. Furthermore, when the best fitted models were used to predict the D, 2D and 4D values, the inactivation in mussel matrix required more time, except for MS2 in mussel heated at 60°C (Table 3-3 and 3-4), showing that the NoV and MS2 were more susceptible to heat treatment in buffered media than in mussel. A similar trend was also observed from the specific z curves (Figure 3-7) generated from the D values of the Biphasic model, where NoV has higher predicted D values than MS2, and thermal inactivation in mussel required more time than in buffered medium, at each temperature studied.

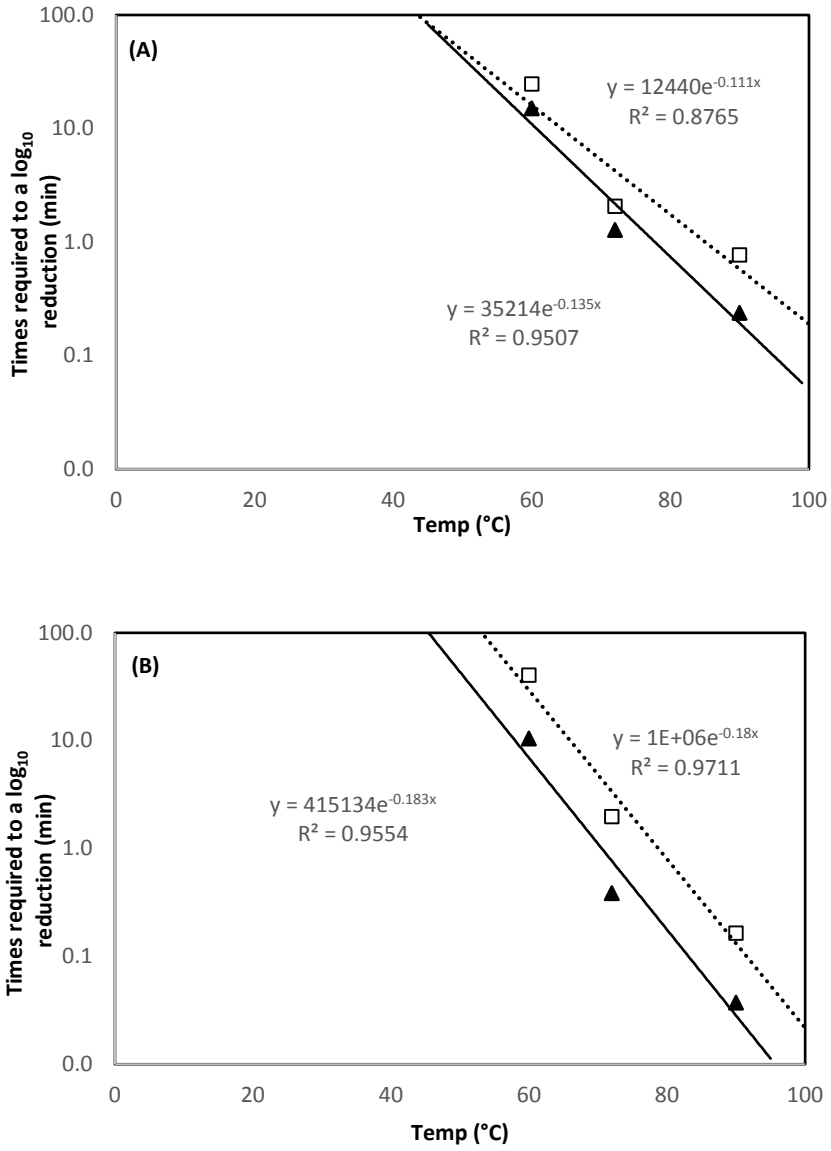


Figure 3-6. Predicted general z curves in buffered media (—) and mussel matrix (⋯) of NoV (A) and MS2 (B) in buffer (▲) and mussel matrix (□) at different temperatures.

Under the assumption that the matrix effect is constant for each temperature, the calculated D values of MS2 was better predicted by the general z curves (Figure 3-6), while for the NoV, the calculated D values from the Biphasic model (Figure 3-7) produced a better prediction.

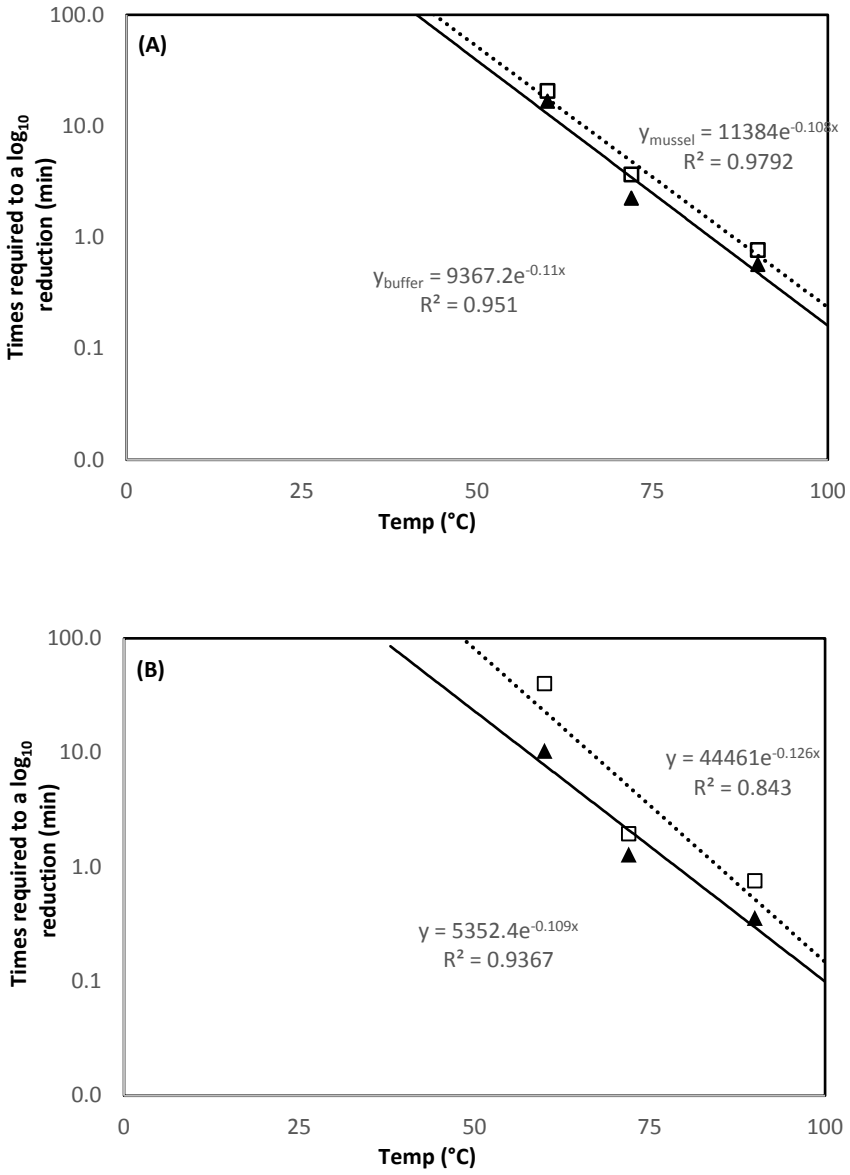


Figure 3-7. Predicted specific z curves in buffered media (—) and mussel matrix (⋯)of NoV (A) and MS2 (B) in buffer (▲) and mussel matrix (□) at different temperatures.

3.4. Discussion

Enteric viruses that caused foodborne diseases are often linked to three categories of food, *i.e.* filter feeder shellfish (bivalve mollusc), raw products contaminated with water containing viruses, and meals or foods prepared by infected food handler (Deboosere *et al.*, 2004b). Thermal inactivation including cooking, pasteurization, sterilisation, canning and blanching has been widely applied in food production systems to reduce or eliminate pathogenic bacteria and viruses (Bertrand *et al.*, 2012; Richards *et al.*, 2010; Teixeira, 2015), thus the determination of D and z values became key

elements in measuring heat resistance of microorganism during thermal inactivation process (Holdsworth *et al.*, 2016; Van Asselt & Zwietering, 2006). In the past 40 years, thermal inactivation has been evaluated for its efficacy to reduce HAV, rotavirus, PV and, NoV and its surrogates in shellfish (Abad *et al.*, 1997; Araud *et al.*, 2016; Bozkurt *et al.*, 2014b; DiGirolamo *et al.*, 1970; Hewitt & Greening, 2006; Millard *et al.*, 1987). However, the use of predictive modelling to predict the D values of the virus in thermal inactivation studies especially in shellfish has just started in the 2010's (Araud *et al.*, 2016; Bozkurt *et al.*, 2015a; Bozkurt *et al.*, 2014b; Park & Ha, 2015; Park *et al.*, 2014). None of these studies, however, were directly compared the predicted D values of NoV and its surrogate, and/or utilised MS2 as NoV surrogate for the inactivation studies.

Predictive inactivation models of NoV and MS2 as its surrogate in different temperatures and matrices were evaluated in this study. The heat treatment at 60, 72 and 90°C mimicked to cooking process of stir-frying, steaming and boiling, respectively. By utilising both linear and non-linear models to fit the viral inactivation curves in this study, the survival data of both viruses during thermal treatment appeared to be best fitted by Weibull or Biphasic than the log linear. This finding is in agreement with some previous studies (Araud *et al.*, 2016; Bozkurt *et al.*, 2013, 2014a) which suggested that Weibull or Biphasic model produced a better fit of thermal inactivation kinetics of NoV and its surrogate, with lower RMSE values than the linear model. Although the Weibull model was appropriate to present the thermal inactivation curves, however this model was unsuccessfully to predict a complete NoV elimination for extended contact time (outside the full duration of the treatment) in both matrices. Thus in this study, only the Biphasic model can be used to predict the required time to complete elimination of NoV. Based on the predicted inactivation curves from Biphasic model (Figure 3-2 and 3-4), for example, a complete elimination of NoV in buffered media and mussel matrix can be achieved after heating at 90°C for approximately 40 and 50 min, respectively.

This present study observed the tailing phenomenon in all curves generated from the inactivation data in both matrices (Figure 3-2 to 3-5). Similar observations were shown from the previous viral

inactivation studies in suspension or shellfish matrix (Araud *et al.*, 2016; Bozkurt *et al.*, 2013; Bozkurt *et al.*, 2015a; Escudero-Abarca *et al.*, 2014; Tuladhar *et al.*, 2012), where tailing phenomenon was present during thermal inactivation. This phenomenon can be hypothesised due to the presence of subpopulations that have a different response toward thermal treatment. The tailing can be caused by the slow reduction of a subpopulation, such as the aggregated viral fraction (Langlet *et al.*, 2007; Tuladhar *et al.*, 2012) or the protected viral particles attached inside the tissue that were more resistant than other subpopulations outside the tissue towards environmental changes due to high content of fat and protein in the tissue (Bidawid *et al.*, 2000). Viral aggregation is potentially occurred due to the changes of the environmental conditions (such as the presence of salts, cationic polymers or suspended organic matters) (Gerba & Betancourt, 2017). Hence, it is worth noting that the use both an aggregated and non-aggregated viral particle in the future studies of inactivation by heat treatment is necessary.

The suitability of MS2 as a NoV surrogate for thermal inactivation study was evaluated in this study by comparing the D, 2D, 4D as well as the z values of NoV and MS2 predicted from the best fitted of three models (Log linear, Weibull and Biphasic). As observed, NoV was generally more resistance to heat than MS2 in both matrices. NoV presented higher z values as well as the D, 2D and 4D values than MS2 in each heating treatment, except for 60°C treatment in mussel (Table 3-3 and 3-4). For example, the z values of NoV and MS2 from thermal inactivation in mussel were 20.75° and 12.79 °C, respectively, and the D values of NoV and MS2 in buffered media at 72°C were 1.28 and 0.39 min, respectively. These observations show evidences that MS2 may not suitable to be used as NoV surrogate to describe the heat resistance of NoV toward thermal inactivation in buffered medium. However, when comparing these results with result from other studies, the thermal resistance of MS2 in suspension at 72°C was similar to HAV (Hewitt *et al.*, 2009), but higher than FCV and MNV-1 (Bozkurt *et al.*, 2014a; Cannon *et al.*, 2006). The predicted D value of MS2 from this study was 0.39 min, while the D values of HAV, FCV and MNV-1 at 72°C were ≤0.30, between 0.10 to 0.12 and 0.09 to 0.17 min, respectively. Hence, MS2 is more relevant to represent the heat-resistance of HAV than

NoV towards thermal inactivation in the suspension, and is potentially to be used as a HAV surrogate.

This present study also evaluates the matrix effect on thermal inactivation by comparing the D, 2D and 4D values of the viruses, predicted from the best fitted of three models in buffered media and bioaccumulated mussel. The differences in thermal resistance of NoV or MS2 in buffered media and in mussels were observed in this study. The D, 2D and 4D values of NoV or MS2 in mussel matrix were higher than in buffered media, except for the D values of MS2 in mussel at 60°C treatment (Table 3-3 and 3-4) where shoulder phenomenon was observed during the first 70 min of contact time (Figure 3-5 A). The time differences to obtain certain log reductions of the virus in buffered media and mussels indicates the occurrence of matrix effect during thermal inactivation, in which NoV or MS2 were more resistance to heat in complex than in simple matrix. This finding is in consistency with result from previous study by Park and colleagues (2014), the virus (MNV-1) was more resistance to heat in complex matrix (dried mussels) than in the simple matrix (culture medium/suspension) at 60, 85 and 100°C treatment which was shown by the higher D values in dried mussels than in suspension. Moreover, similar trend was also observed from a study by Croci and colleagues (2012), where the number of infectious of NoV and FCV from heating at 80°C for 3 to 15 min were higher in complex matrix (spiked mussels) than in simple matrix (viral suspension). Possible explanation for this matrix effect is that the mussel contains protein and fat which could protect the viral particle from the heat (Bozkurt *et al.*, 2014b) and prevent viral aggregation (Croci *et al.*, 2012).

3.5. Conclusions

Overall, this study presents tailing phenomena during thermal inactivation of NoV and MS2, which due to the occurrence of heat-resistant subpopulation. Thus, non-linear models (Weibull and Biphasic) were more appropriate than linear model (log linear) to describe the inactivation kinetics of both viruses. The Biphasic model was also more suitable than Weibull to predict virus survival for

extended contact times (outside the full duration of the treatment), when two or four log reductions are considered as the thermal inactivation objective. The thermal inactivation kinetics were affected by different matrices, where complex matrix such as mussel provided higher protection for the viral particles against heat treatment than the simple matrix (buffered media). It is worth noting that MS2 can be used as NoV surrogate to describe this phenomenon, but caution should be taken when extrapolating the MS2 inactivation kinetics for NoV inactivation studies because MS2 is less resistant than NoV toward thermal treatment.

Chapter 4. Chlorine dioxide inactivation of NoV and MS2 in buffered media and artificially contaminated Tasmanian Blue Mussels (*Mytilus galloprovincialis*) tissue

4.1. Introduction

Consumption of raw or improperly cooked shellfish has been identified as a major cause of of NoV infection (Alfano-Sobsey *et al.*, 2012; Maunula & Von Bonsdorff, 2014). Bitler *et al.* (2013) suggested that the attack rate (which is defined as the number of cases per 100,000 persons exposed to NoV contaminated food) in shellfish was the highest amongst other type of foods (produce and ready to eat foods). Food in general are contaminated by NoV through different routes, such as contact with infected food handlers or cross-contamination during food processing (Hall *et al.*, 2012); or contact with NoV-contaminated water at their harvesting/growing sites during production (Bellou *et al.*, 2013; Polo *et al.*, 2015; Rodríguez-Lázaro *et al.*, 2012). While contamination of NoV into water environment can be caused by several factors, such as sewage leak, surface contamination due to heavy rainfall or flooding, water treatment (chlorination) failure and water system breakdown (Maunula, 2007). Therefore, the use of untreated contaminated-water for food processing and handling could contribute to NoV contamination in food.

In the case of potentially transmission of NoV during the food processing, the implementation of GHP and the application of disinfectants and sanitizers play important role in reducing the contamination (Barker *et al.*, 2004; Boxman, 2013). Many studies have highlighted the potential application of disinfectants to reduce viral contamination during food processing and to be used as a cleaning agent for the processing facilities, particularly using NoV surrogates (D'Souza & Su, 2010; Feliciano *et al.*, 2012; Fraisse *et al.*, 2011; Grove *et al.*, 2015; Malik & Goyal, 2006; Takahashi *et al.*, 2011). Among other disinfectants, these studies showed that chlorine-containing compounds such

as sodium hypochlorite, chloramines and chlorine dioxide (ClO_2) have effectively reduced viral contamination.

Chlorine-containing compounds have been considered and reviewed by the expert panels of FAO/WHO as potential disinfectants used in food production and processing globally (FAO & WHO, 2009) and have been widely used as disinfectants in the cleaning and sanitation steps of seafood processing (Huss, 1994). For instance, chlorine (one of these compounds) is a common disinfectant added into water which is used for different purposes, including to wash the fish, to make the ice for chilling the fish, to thaw the frozen fish or to cool the canned fish after retorting (FAO & WHO, 2000). Hypochlorite is also used to decontaminate containers and table surface in the fish processing industry in Indonesia with concentration ranges from 20 to 100 mg/l of total chlorine (FAO & WHO, 2009). From Indonesia perspective, the use of chlorinated-water in fish production lines in Indonesia is regulated through the Decree of Ministry of Marine and Fisheries Affairs (MMAF) KEP 01/MEN/2002 about the Intensive Quality Management System of Fishery Product (MMAF Indonesia, 2002), where chlorine can be added into water as a disinfectant for washing purpose at the maximum of 10 mg/l of total chlorine (MMAF Indonesia, 2002). Moreover, the free chlorine residue in the water to be used in fish processing should not exceed 5 mg/l (Ministry of Health Indonesia, 2010).

The efficacy of chlorine-containing compounds to inactivate and to reduce enteric virus such as NoV and its surrogates (*e.g.*, FCV, MS2 phage, MNV and PV-1), has been investigated and evaluated (Feliciano *et al.*, 2012; Kim *et al.*, 2012; Kitajima *et al.*, 2010; Montazeri *et al.*, 2017; Rachmadi *et al.*, 2018; Sigstam *et al.*, 2014; Tung *et al.*, 2013). Results from these studies showed that the difference in disinfectants efficacies to reduce and to inactivate viruses were observed. The variability in the disinfectant efficacies of those studies were being influenced by some parameters used during the inactivation, such as: the differences in mode of inactivation, types and concentration of the disinfectant, contact time and virus species.

Another factor that may influence the disinfection efficacy is the differences in disinfection decay rate (*k'* values) (Haas & Joffe, 1994; Shin & Sobsey, 2008) which occurs when different types of chlorine-containing compounds (such as hypochlorite, chloramines and ClO_2) and different modes of inactivation that are being used (Gómez-López *et al.*, 2009). The efficacy of chlorine-containing compounds as disinfectant is also influenced by pH, temperature and the presence of organic matter (Hirneisen *et al.*, 2010; Kingsley *et al.*, 2014; Morino *et al.*, 2009; Tung *et al.*, 2013).

In particular, previous studies that evaluated the efficacy ClO_2 to reduce the NoV and its surrogates were only performed in suspension or buffered media, produce or fruit matrices and in hard surfaces, and rarely compared it with meat matrix, especially shellfish (Girard *et al.*, 2016; Kingsley *et al.*, 2018; Lim *et al.*, 2010; Montazeri *et al.*, 2017; Morino *et al.*, 2009; Yeap *et al.*, 2016). Compared to the matrices used in those studies, shellfish has different composition of both organic and inorganic compounds. As a consequence, the application of ClO_2 as disinfectant in the shellfish matrix may represent a different efficacy than the result from the previous studies on chlorine-containing compounds disinfection. Hence, investigating the efficacy of ClO_2 as disinfectant in shellfish matrix is required.

The efficacy of the treatment is commonly assessed by calculating the concentration of ClO_2 over the time (*C*) and the decay rate (*k'*) values using the first-order kinetic, and followed by predicting the inactivation kinetics using the Hom model (Haas & Joffe, 1994; Hornstra *et al.*, 2011). This approach has been widely used to calculate the efficacy of chlorination as well as ClO_2 treatment to reduce microbial and viral contamination in water treatments (Cromeans *et al.*, 2010; Haas & Joffe, 1994; Hornstra *et al.*, 2011; Jacangelo *et al.*, 2002; Kahler *et al.*, 2010; Murphy *et al.*, 2014). Another model such as the modified biphasic can also be used for the comparison of the inactivation kinetic and to describe the tailing phenomenon during the ClO_2 inactivation in drinking water (Hornstra *et al.*, 2011).

In our study, the efficacy of ClO_2 treatment to reduce NoV and MS2 bacteriophage were evaluated in both buffered media and artificially-contaminated mussel. Pre-treatment RT-qPCR was used to enumerate the infectious NoV from the treatment, while plaque assay method was used for the quantification of MS2. In the same ClO_2 treatment, the reliability of MS2 bacteriophage as a NoV surrogate was also assessed by comparing the inactivation kinetic of both viruses in the same matrix, while the matrix effect was evaluated by comparing inactivation kinetic of the virus in buffered media and mussel. The quasi-mechanistic Hom, Weibull and Biphasic model were used to calculate the inactivation kinetics of both viruses during the treatment, while the first-order kinetic equation was used to determine the decay rate of ClO_2.

4.2. Materials and methods

4.2.1. Mussels preparation and artificial contamination.

Five kilograms of live Tasmanian Blue Mussel (*Mytilus galloprovincialis*) were purchased from a single local fish market/supplier. Mussel acclimatisation and depuration were done as described in Section 3.2.3 for 6 h and by changing the sterilized sea water every 2 h. One hundred pieces of tissue mussels were taken out from the shells and were pre-washed with sterile saline water (0.3% NaCl) at 4°C. Artificial contamination of the mussel was done by dipping the tissue in NoV and MS2 solutions at a final concentration of approximately 10^8 copies/ml and 10^8 PFU/ml, respectively, for 30 min. The tissues were then drained for 60 min at 4°C to remove the excessive solution. The dipping method was done to provide NoV contamination at the shellfish tissue surface (not inside the tissue) which mimicked the process of viral cross-contamination by secondary transmission.

4.2.2. Chlorine dioxide treatments

Chlorine dioxide (ClO_2) stocks (5,000 ppm) were prepared following procedure from Cleanoxide® (NaturalWater Solutions, Australia) by mixing 1 part of solution A and 9 part of solution B. The mixture was shaken for 15 s and stored at a dark glass bottle for 8-10 h in 4°C to complete the

reaction. The concentration of total ClO_2 stock was determined by the DPD-based method (Palin, 1957) using the Palintest Chlorometer ClO_2+ Kit (Palintest, Australia). This kit was able to quantify only chlorine dioxide in the sample. The ClO_2 stock was serially diluted to make 250; 500; and 1,000 ppm of working solutions. The ClO_2 treatments were performed in two different matrices *i.e.*, buffered medium (PBS) and mussel matrices at different ClO_2 concentration (10; 20 and 40 ppm) for certain period of times as shown in Table 4-1. The treatment of each concentration in both matrices were done in triplicate and carried out in 50 ml plastic tube incubated at water bath to maintain the temperature at 20°C. For the treatment in buffered media, tube containing 45 ml of PBS-ClO_2 suspension were prepared by adding the ClO_2 working solutions into the 40 ml PBS solution in the tube to obtain the final concentrations of 10; 20; and 40 ± 1 ppm. A five ml of virus stock containing NoV and MS2 at concentration of 10^7 copies/ml and 10^8 PFU/ml, respectively, were added into the tubes. The concentration of ClO_2 was measured immediately following procedure from the manufacture (Palin Test Kit, Australia) after certain exposure time (Table. 4-1). For the ClO_2 treatment in mussel matrix, 5 g of contaminated-mussel was dipped into the plastic tube containing 45 ml ddH_2O-ClO_2 and exposed to the treatment for certain periods of time, as shown in Table 4-1. After each exposure time, the ClO_2 concentration was measured immediately, and the mussels were transferred to a new tube and added with 1 ml of 10% sodium thiosulfate to inactivate the remaining ClO_2. The sample was then concentrated and purified as described in Section 4.2.4.

Table 4-1. Exposure time of ClO_2 treatment at different concentrations

ClO_2 concentration (ppm)	**Σ treatment tubes**		**Exposure time (min)**
	Buffered medium	**Mussel**	
0 (Initial/No treatment)	3	3	0
10	21	21	1; 10; 20; 30; 40; 50; and 60
20	21	21	1; 10; 20; 40; 60; 80; and 100
40	21	21	1; 20; 40; 80; 120; 160; and 200

4.2.3. Analysis of ClO_2 residue by Palintest kit

The remaining ClO_2 in the suspension after each exposure time was quantified using the Palintest Kit (Australia) according to the manufacturer's instructions without any modifications.

4.2.4. Virus and bacteriophage purification

The infectious viruses from the treatment in mussel matrix were purified following the procedure of Lewis and Metcalf (1988) and Mullendore *et al.* (2001), with modifications as previously described in Section 3.2.6.1 of this thesis, while the infectious viruses in buffered media were directly processed for subsequent plaque assay (for MS2) or pre-treatment and RNA extraction (for NoV) without the purification step.

4.2.5. Enumeration of MS2 by plaque assay

The infectious MS2 from the treatment was enumerated using a double layer agar method (EPA, 2001), with modifications, as previously described in Section 2.2.2.2.a of this thesis.

4.2.6. Virus pre-treatment and RNA extraction

Prior to nucleic acid extraction, the purified sample was pre-treated using RNase as previously described in Section 2.2.4 of this thesis. Subsequently, RNA samples was extracted using method of Chomczynski and Sacchi (2006), with modifications, as previously described in Section 2.2.2.2.b of this thesis

4.2.7. Enumeration of NoV by RT-qPCR

For the enumeration of infectious NoV GII, the RT-qPCR were performed using method of Jothikumar *et al.* (2005) with modifications as previously described in Section 3.2.6.3 of this thesis.

4.2.8. Modelling and statistical analysis of ClO_2 inactivation kinetics

As described from previous study by Haas and Joffe (1994), the decay rate of ClO_2 during inactivation process was calculated using a first-order kinetic equation as follows.

$$C = C_0 e^{-k\prime t} \quad \text{(Equation 4-1)}$$

where *C* and C_0 are ClO_2 residue (mg/l) at time *t* and time 1 min (closest measurement to time zero), respectively, and *k'* is the first-order ClO_2 decay rate constant (min^{-1}). The *k'* value for each experiment were calculated using the Solver function in Microsoft Excel 365 (Microsoft Corp).

The infectious NoV and MS2 from each treatment were fitted into Hom, Weibull and Biphasic models. The Hom model equation as previously described by Thurston-Enriquez *et al.* (2003) and Haas and Joffe (1994) is written as follows.

$$Ln\left(\frac{N}{N_0}\right) = -kC_0 t^m \, x \left(1 - e^{(-nk\prime t/m)}/(nk't/m)\right) \quad \text{(Equation 4-2)}$$

where Ln (N/N_0) is the natural log of the survival ratio of virus (number of viruses remaining at time *t* (*N*) divided by the average of initial number of viruses without treatment (N_0)). The *k* value is the Hom inactivation rate constant, *n* is the dilution coefficient, and *m* is an empirical constant that describes the deviation from ideal Chick-Watson kinetics (Sigstam *et al.*, 2014). The Solver function in Microsoft Excel 365 (Microsoft Corp.) was used to determine the values for each model's coefficients by minimising the sum of squares of the difference between the observed and predicted of natural log reduction over the time ($Ln(N/N_0)$) for viral inactivation with the same virus and matrix. Inactivation curves of NoV and MS2 (log reduction over the time (min) ($Log_{10}(N/N_0)$) were also calculated and created using Microsoft Excel 365 (Microsoft Corp.).

Weibull (Eqn. 3-3) and Biphasic model (Eqn. 3-5) previously described in Section 3.2.7 were used to predict the log reduction value over the time ($Log_{10}(N/N_0)$) for each experiment with the same virus and matrix. The inactivation curves of the models were fitted and calculated using GInaFiT for Microsoft Excel (Geeraerd *et al.*, 2005). The coefficient of determination (R^2) was used to evaluate

the predicted *C* values compared to the observed, while the Root Mean Square Error (RMSE) values was used to measure the goodness-of-fit of the decay rate and the inactivation models.

The single factor of Analysis of Variance (ANOVA) was carried out to calculate the differences of the calculated *k'* rate and the observed $\log_{10}$ reductions of NoV and MS2 using Real Statistics Add-ins for Microsoft Excel 365 (Microsoft Corp).

4.3. Results

4.3.1. ClO_2 decay in buffered media and mussel matrix

The residue of chlorine dioxide (ClO_2) (at pH 6.9 ± 0.2) of each treatment at 20 ± 1°C over the inactivation period in buffered media is shown in Figure 4-1. The ClO_2 residue analysis was done using Palintest kit with the detection limit (LOD) of 0.02 ppm. The residue values were plotted against the exposure time to produce ClO_2 decay curves. In general, ClO_2 concentration decreased over time during the treatment. These curves were then fitted using the first-order kinetics model to calculate the initial concentration residue (C_0) and the decay rate (k') of ClO_2. The calculated (C_0) in the solution at 1 min exposure (the closest measurement to time zero) were 8.40, 19.25 and 30.13 ppm for treatment with 10, 20 and 40 ppm, respectively. The k' of ClO_2 during treatment were varied between 0.052 to 0.056 min^{-1}, with the average of 0.053 ± 0.023 min^{-1}. The R^2 between the observed and the predicted *C* values for 10; 20; and 40 ppm treatment were above 95% for each treatment with the RMSE values of 0.516; 0.834; and 2.338, respectively.

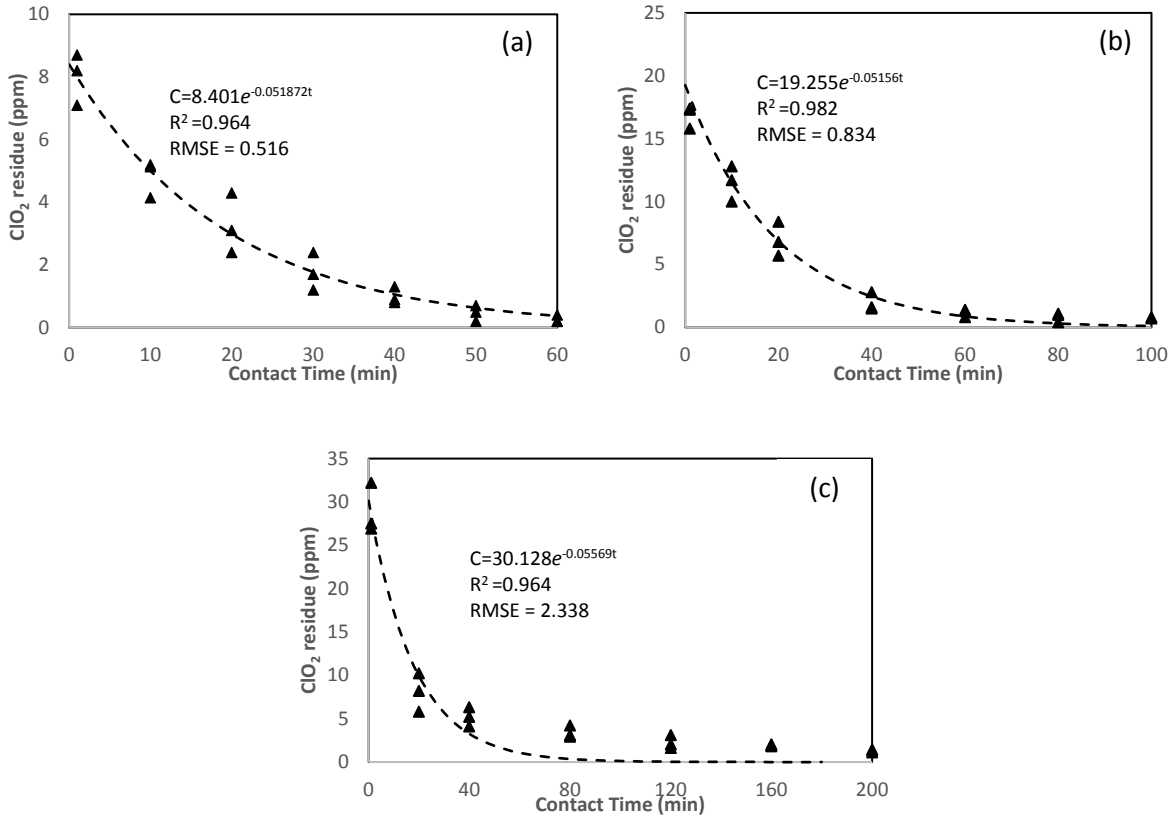


Figure 4-1. The observed (▲) and predicted (---) values of ClO_2 residue (*C*) (from (a) 10, (b) 20, and (c) 40 ppm treatment at 20°C for different exposure times in buffered media.

Figure 4-2 describes the effect of mussel matrix on ClO_2 concentration over the time. A decrease in ClO_2 concentration was observed in all cases, similar to the apparent effects in the buffered media. However, the ClO_2 decay in mussel matrix showed higher *k'* value than in the buffered media. The average *k'* rate in mussel (0.080 ± 0.0024 min^{-1}) was significantly higher ($p<0.01$) than in buffered media (0.053 ± 0.0023 min^{-1}). Moreover, the observed initial ClO_2 concentrations (C_0) of 10, 20 and 40 ppm treatment in mussel matrix (7.40, 12.51 and 17.86 ppm, respectively) were lower than in buffered media. Similar observation to the ClO_2 decay curves in buffered media, the R^2 values between observed and predicted *C* in those three curves (10; 20 and 40 ppm) in mussel matrix were more than 95% with the RMSE value of 0.513, 0.606, and 1.335, respectively. In addition, the observed residue values of ClO_2 from all treatments in both matrices (buffered media and mussel) after 60 min exposure were less than 5 ppm.

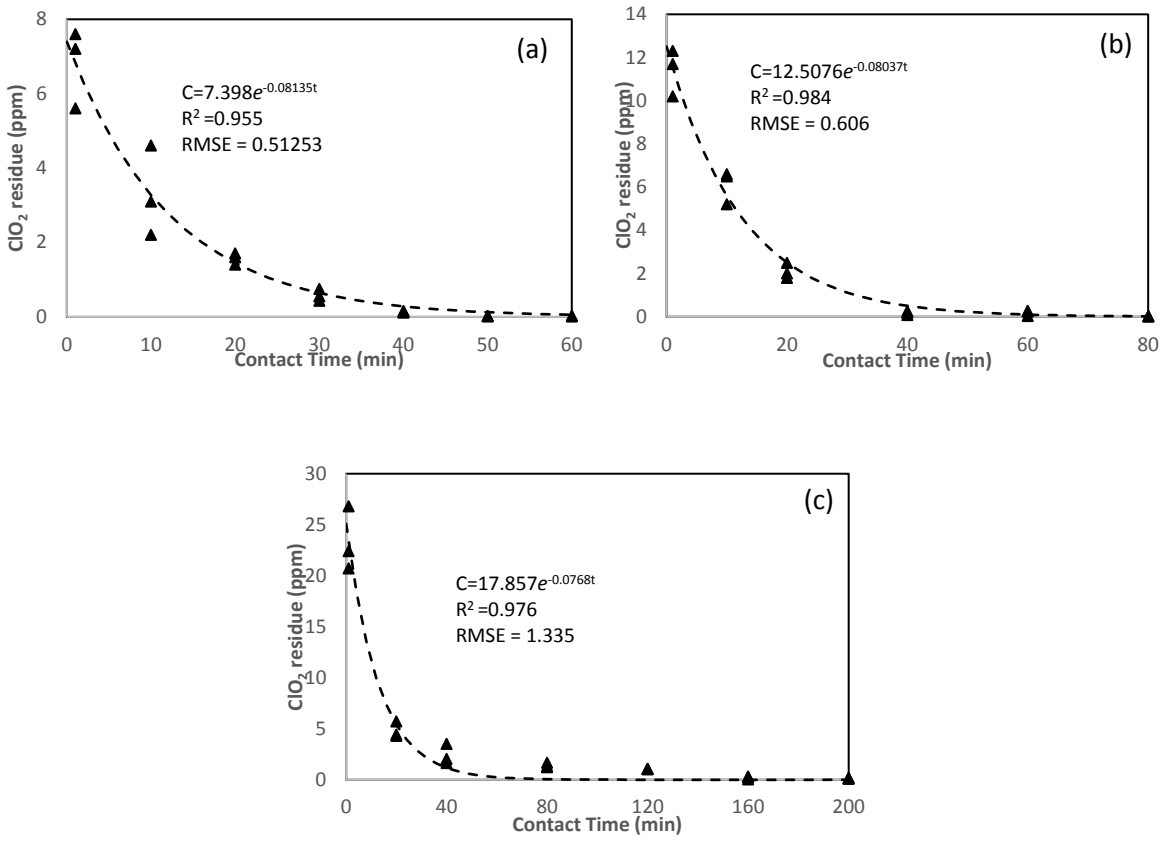


Figure 4-2. The observed (▲) and predicted (---) values of ClO_2 residue (*C*) from (a) 10, (b) 20, and (c) 40 ppm treatment at 20°C for different exposure times in mussel matrix.

4.3.2. The efficacy of ClO_2 treatment on NoV and MS2 in buffered media

The infectious NoV in buffered media treated with different concentrations of ClO_2 at 20 ± 1°C were enumerated using pre-treatment RT-qPCR assay with LOQ at 250 copies/ml (2.40 log_{10} copies/ml). To calculate the reductions of MS2, the infectious MS2 in buffered media after exposure to ClO_2 treatment were analysed using plaque assay (LOQ at 20 PFU/ml). The observed log_{10} reductions of the viruses ($log_{10}(N/N_0)$) were plotted against the contact times to generate inactivation curves. In general, the viral inactivation curves observed in this study showed a tailing shape, thus non-linear models were better to describe the viral inactivation by ClO_2. The inactivation curves of NoV and MS2 in buffered media fitted using non-linear models *i.e.*, Hom, Weibull and Biphasic models were shown in Figure 4-3 and 4-4. Since the Hom model produced the reductions in natural logarithm value ($Ln(N/N_0)$), therefore the value of log reductions ($log_{10}(N/N_0)$) was obtained by extrapolating the value of $Ln(N/N_0)$ using Microsoft Solver Add-in.

Table 4-2. The RMSE and R^2 values of the ClO_2 inactivation models of Hom, Weibull and Biphasic

Matrix	Virus	Initial viral conc. (N_0)	ClO_2 (ppm)	Inactivation model					
				Hom		Weibull		Biphasic	
				RMSE	R^2	RMSE	R^2	RMSE	R^2
Buffered media	NoV	6.39 ± 0.20 log_{10} copies/ml	10	**0.121**	0.922	0.131	0.922	0.123	0.935
			20	**0.316**	0.793	0.349	0.789	0.324	0.830
			40	**0.451**	0.856	0.511	0.852	0.473	0.884
	MS2	7.37 ± 0.11 log_{10} PFU/ml	10	**0.128**	0.960	0.139	0.959	0.142	0.960
			20	0.330	0.922	0.369	0.917	**0.305**	0.946
			40	0.397	0.955	0.722	0.871	**0.355**	0.971
Mussel	NoV	6.59 ± 0.44 log_{10} copies/g	10	**0.145**	0.725	0.193	0.582	0.161	0.725
			20	**0.261**	0.641	0.283	0.640	0.273	0.685
			40	**0.213**	0.830	0.232	0.827	0.223	0.848
	MS2	6.40 ± 0.07 log_{10} PFU/g	10	**0.172**	0.784	0.195	0.761	0.177	0.813
			20	0.139	0.945	0.192	0.907	**0.136**	0.956
			40	**0.211**	0.895	0.261	0.861	0.212	0.913

Note: *The RMSE values written in bold indicate the lowest RMSE produced by the best-fitted model.*

Hom's showed the lowest RMSE values amongst the other models in predicting the log_{10} reductions value of NoV for each treatment (Table 4-2), thus these predicted log reductions values of Hom's were used to describe the treatment efficacy in NoV. The highest NoV reduction predicted in buffered media treated with ClO_2 for 60 min was observed from the 40 ppm ClO_2 with 3.05 log_{10} reductions, while treatment at 10 and 20 ppm were predicted to reduce NoV numbers by 1.61 and 2.38 log_{10} reductions, respectively. In contrast with the NoV inactivation data, Biphasic model was observed as the best fitted model (giving the lowest RMSE values) to predict the log_{10} reductions of MS2 inactivated by 20 and 40 ppm ClO_2 in buffered media (Table 4-2.), while Hom only gave the best prediction at 10 ppm treatment. Hence, the predicted log_{10} reductions of MS2 in 20 and 40 ppm treatment were calculated using Biphasic model, while the predicted log_{10} reductions of 10 ppm treatment was calculated using Hom model. Compared to NoV, similar observation was shown on the efficacy of ClO_2 treatment, where the higher ClO_2 concentration produced the higher value of estimated log reductions. However, MS2 were more susceptible towards ClO_2 treatment, where the log_{10} reductions of MS2 in the same treatment for the same time exposure were higher than NoV. The predicted reductions of MS2 in 10, 20 and 40 ppm were 2.46, 4.02 and 5.18 log_{10} reductions, respectively.

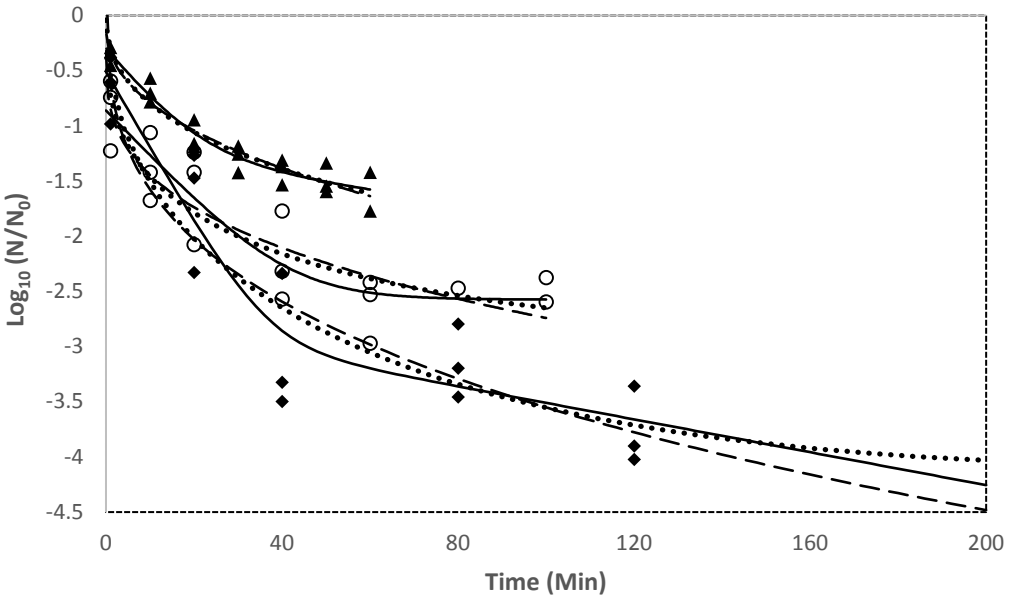


Figure 4-3. The log reductions ($Log_{10}(N/N_0)$) curves of NoV in the buffered media fitted using Hom (···), Weibull (---), and Biphasic model (—) treated with 10 (▲), 20 (○), and 40 (◆)ppm ClO_2 for different exposure times

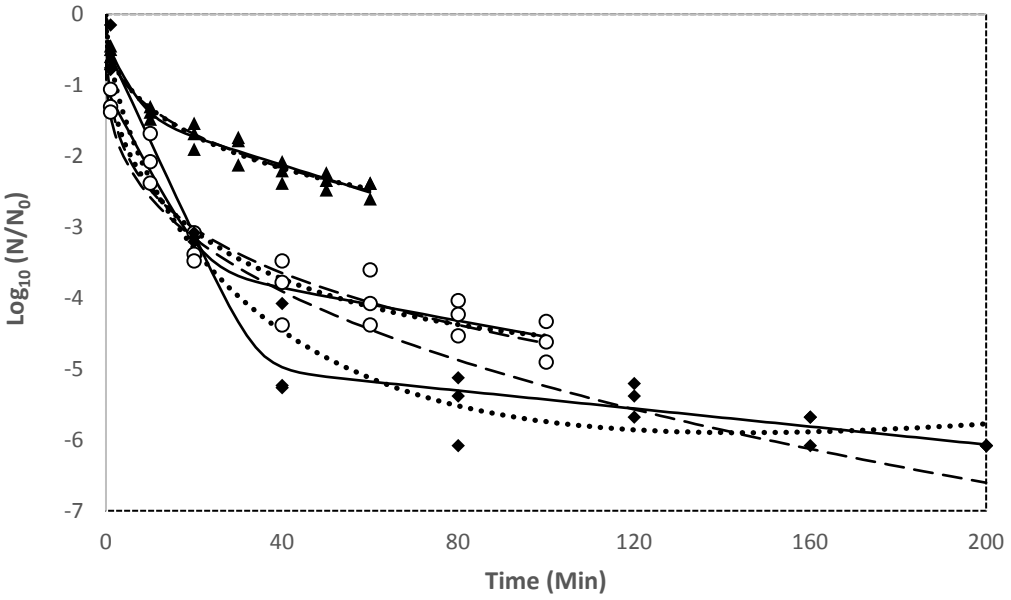


Figure 4-4. The log reductions ($Log_{10}(N/N_0)$) curves of MS2 in the buffered media fitted using Hom (···), Weibull (---), and Biphasic model (—) treated with 10 (▲), 20 (○), and 40 (◆) ppm ClO_2 for different exposure times

4.3.3. The efficacy of ClO_2 treatment on NoV and MS2 in mussel matrix

The viral inactivation curves by ClO_2 in the mussel matrix are presented in Figure 4-5 and 4-6. In the mussel matrix experiment, Hom model produced better prediction on the viral reduction treated

with 10, 20 and 40 ppm ClO_2 than Weibull or Biphasic model, except for MS2 treated with 20 ppm ClO_2 (where the biphasic was the best-fitted model for this treatment) (Table 4-2). The predicted reduction values of NoV in mussel treated with 10, 20 and 40 ppm of ClO_2 for 60 min were 1.14, 1.38 and 1.43 log_{10} reductions, respectively. These values were lower than the MS2 reductions toward similar treatments, except in the 10 ppm treatment. The MS2 reduction treated with ClO_2 at concentration of 10, 20 and 40 ppm for 60 min were 1.09, 1.66 and 1.72 log_{10} reductions.

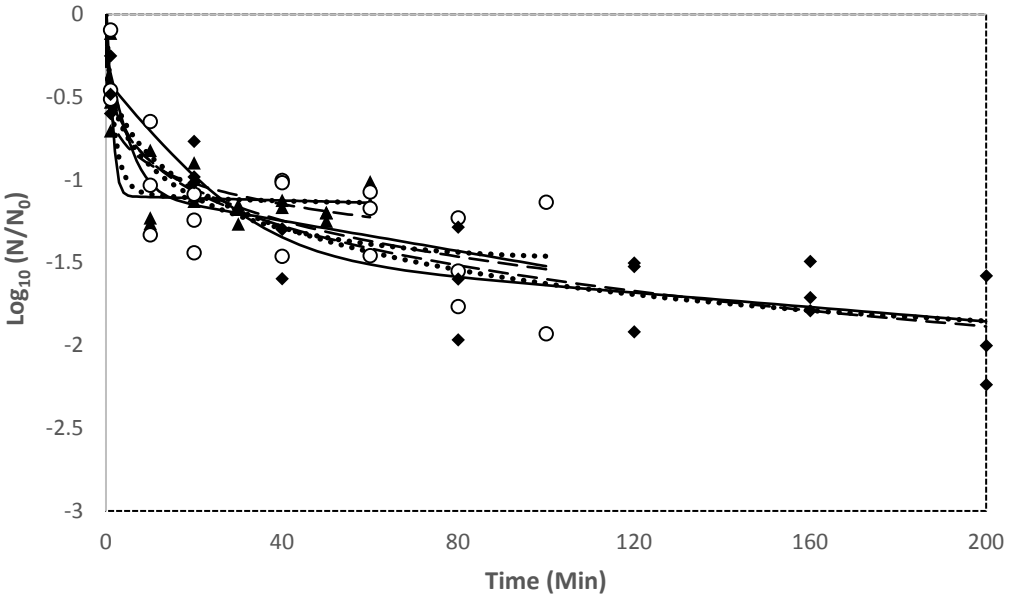


Figure 4-5. The log reductions ($Log_{10}(N/N_0)$) curves of NoV in the mussel fitted using Hom (⋯), Weibull (---), and Biphasic model (—) treated with 10 (▲), 20 (○), and 40 (◆) ppm ClO_2 for different exposure times

Although the predicted log_{10} reductions of NoV was slightly higher than MS2 in the 10 ppm treatment for 60 min in mussel matrix, however, the result from analysis of variance (ANOVA) showed that there were no significant differences ($P>0.05$) between the average of observed log_{10} reductions of NoV (≈1.07 ± 0.07 log_{10} reductions) and MS2 (≈1.08 ± 0.12). From the observed data, the maximum reduction of NoV and MS2 in mussel matrix were achieved by treated using 40 ppm ClO_2 for 200 min. The average of NoV and MS2 reductions observed from these treatments were 1.94 ± 0.33 and 2.17 ± 0.19 log_{10} reductions, respectively (Figure 4-5). In overall, MS2 were more susceptible than NoV toward ClO_2 treatment in the mussel matrix which were similar to the

observed trend in the experiment using buffered media. This finding indicated that the MS2 is not the best candidate for a NoV surrogate in this study since the number of infectious MS2 does not represent the infectious NoV from the treatment.

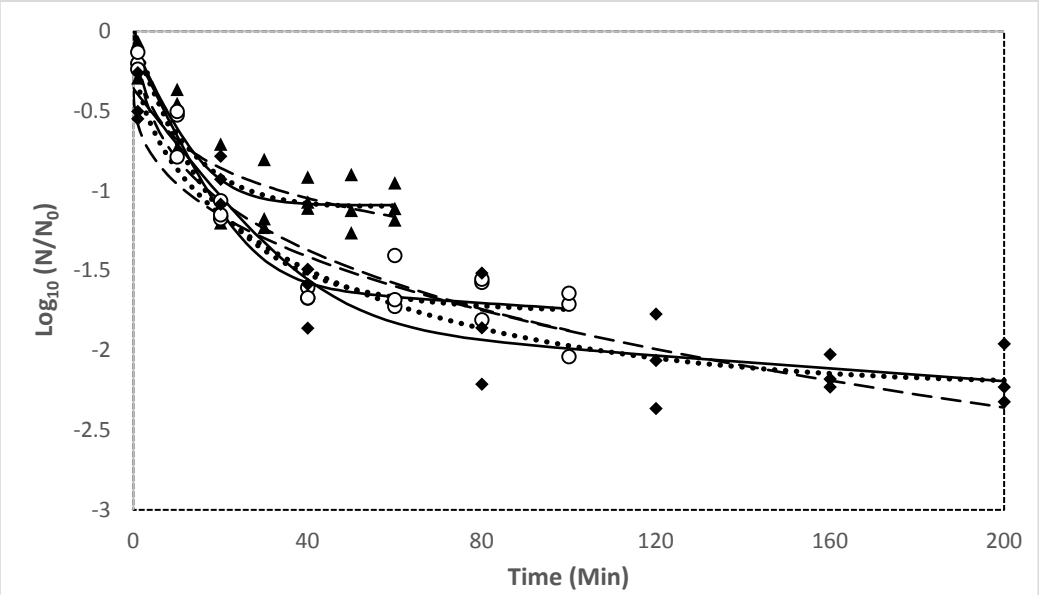


Figure 4-6. The log reductions ($Log_{10}(N/N_0)$)curves of MS2 in the mussel fitted using Hom (⋯), Weibull (---), and Biphasic model (—) treated with 10 (▲), 20 (○), and 40 (◆) ppm ClO2 for different exposure times

In this study, the matrix effect was observed during ClO_2 inactivation for both viruses at concentration of 10, 20 and 40 ppm. The observed maximum log reductions of NoV and MS2 after exposed to ClO_2 for certain periods in buffered media were significantly higher ($P<0.05$) than in mussel matrix as presented in Table 4-3. For example, the average of NoV and MS2 reduction in buffered media after 100 min treated with 20 ppm were 2.49 ± 0.16 and 4.62 ± 0.29 log_{10} reductions, respectively, where only 1.53 ± 0.56 and 1.80 ± 0.21 log_{10} reductions were observed in the mussel matrix.

Table 4-3. The average of observed maximum reduction of NoV and MS2 treated by ClO_2 exposed for certain periods

Virus	Treatment	The average of $log_{10}(N/N_0)$ (log_{10} reductions)	
		Buffered Media	Mussel
NoV	10 ppm for 60 min	1.60 ± 0.25[a]	1.07 ± 0.07[b]
	20 ppm for 100 min	2.49 ± 0.16[a]	1.53 ± 0.56[b]
	40 ppm for 120 min*	3.76 ± 0.35[a]	1.65 ± 0.23[b]
MS2	10 ppm for 60 min	2.49 ± 0.16[a]	1.08 ± 0.12[b]
	20 ppm for 100 min	4.62 ± 0.29[a]	1.80 ± 0.21[b]
	40 ppm for 200 min	6.08 ± 0.01[a]	2.17 ± 0.19[b]

*Note: *The exposure time of 120 min was used in the 40 ppm treatment, as some missing data was observed in the exposure of 160 and 200 min*
The same letter in the same row denotes no significant differences (p>0.05)

4.4. Discussion

The likelihood of viral transmission to human from the ingestion of food contaminated by enteric viruses were reported from some foodborne outbreaks. Although the major sources of enteric viral contamination in food originated from the main transmission route where the food has been directly contacted with faecal-contaminated water (Bellou *et al.*, 2013), for example in the NoV or HAV contamination in shellfish, some types of food have been reported to be contaminated by enteric viruses through secondary transmission *via* cross-contamination during food handling. Findings from previous studies showed that some enteric viruses and their surrogate can be transmitted into the food through the contact with food handlers' hand, washing water and the equipment during handling and processing in fresh produce, fruit and ready-to-eat meals (Dalton *et al.*, 1996; Grove *et al.*, 2015; Holvoet *et al.*, 2014; Maunula *et al.*, 2013; Schmid *et al.*, 2007). During the viral cross-contamination in food, viral particles were commonly attached in the food surfaces (Todd *et al.*, 2009), thus this contamination can be reduced or eliminated by the application of disinfectant in washing step. In addition, the use of disinfectants as control strategies in GHP and GMP regime have been widely applied in food industries, including for fisheries product (FAO & WHO, 2009).

In fish processing industries, ClO_2 is generally used to improve the application of hygienic practices, rather than in the decontamination procedure (FAO & WHO, 2009). The efficacy of this compound to reduce the level of pathogenic bacteria has been studied in oyster (Shin *et al.*, 2004) and other seafood products (salmon, grouper, scallops, and shrimps) (Kim *et al.*, 1999) as well as in antimicrobial ice used in fish processing (Wang *et al.*, 2010). These studies suggested that ClO_2 can be used as an effective bacterial disinfectant in fish and oyster with the minimum concentration of 20 ppm. However, studies that evaluate the efficacy of this compound against viral contamination in seafood are still limited. Hence in the current study, the efficacy of ClO_2 treatment with various concentration from 10 to 40 ppm was evaluated to reduce NoV in buffered medium and mussel matrix.

As previously described in Chapter 1 and 2, NoV is the most common cause of NoV infection and the challenge to cultivate this virus makes it difficult to perform the quantification using a cell culture system. Therefore, the used of cultivable surrogates such as MNV, FCV and MS2 have been widely proposed to overcome this problem and to understand the inactivation mechanism of NoV. In this study, the efficacy of ClO_2 (at 20 ± 1°C with pH 6.9 ± 0.2) to reduce NoV and MS2 using identical experiment condition in two different matrices (buffered media and mussel) was evaluated and compared. The experiment in mussel matrix was designed to understand the efficacy of ClO_2 to reduce viral particles contaminated the mussel in which cross-contamination scenario was applied, hence the artificial contamination of the virus in this study was performed by dipping the mussel's tissue into viral stock.

Results from this current study showed that the decay of ClO_2 in both matrices was observed during viral inactivation. The ClO_2 decay rates were constant following the model of first-order-kinetic with the R^2 values of >95% in all concentrations observed. The matrix effect in ClO_2 decay was also observed in the inactivation of both viruses (NoV and MS2) where the ClO_2 decay rate in mussel was faster than in buffered media (solution). The possible explanation of this matrix effect is that the mussel tissue contains more organic and inorganic compounds than the buffered media. Thus the

available chlorine including ClO_2 were being consumed faster for oxidation, addition and electrophilic substitution reactions of these compounds (Deborde & von Gunten, 2008) in the mussel matrix than in buffered media. To maintain the ClO_2 concentration during inactivation treatment, a closed reactor (pump) as used by (Sigstam *et al.*, 2014) can be suggested in future inactivation experiment in both matrices.

The virucidal effects of ClO_2 (as disinfectant) in NoV and MS2 were investigated in this study. In viral inactivation studies using disinfectants, a temporary inactivation could occur due to a reversible change in the virus conformation, while damage on the capsid protein and/or nucleic acid may resulted in the permanent inactivation of the virus (Thurman & Gerba, 1988).The efficacy of ClO_2 to inactivate viral particles varied depend on the virus species as well as the matrix used in the experiment. In general, MS2 was more susceptible than NoV towards ClO_2 treatment especially in buffered media. The discrepancies in the effectivity for viral inactivation by chlorine-containing compounds were also observed from previous studies when different viruses were used for the identical treatment in their studies (D'Souza & Su, 2010; Duizer *et al.*, 2004; Dunkin *et al.*, 2017; Kitajima *et al.*, 2010; Montazeri *et al.*, 2017; Shin & Sobsey, 2008; Sigstam *et al.*, 2013). Generally, the virus stability depends on the capsid structure to provide protection from environmental stress (Hirneisen *et al.*, 2010; Nuanualsuwan & Cliver, 2003), thus different capsid structure has different mechanisms toward environmental stress which affect their persistence in the environment and their sensitivity to disinfectants (Cook *et al.*, 2016; da Silva *et al.*, 2007; Seitz *et al.*, 2011; Verhaelen *et al.*, 2013).

It is worth noting that each virus species or strain has a different structure of capsid protein and genome, thus it has a different response toward disinfectant such as chlorine-containing compounds (Sigstam *et al.*, 2013; Wigginton *et al.*, 2012). In addition, from the extensive investigation of the viral inactivation mechanism using MS2, Wigginton and colleagues (2012) suggested that even the same virus species may have different susceptibilities toward environmental stress. As consequence, the observation from the current study together with those previous studies that highlighted the

differences in susceptibility of NoV and MS2 toward ClO_2 raise a concern about the compatibility of MS2 as NoV surrogate. Caution must be considered when utilising inactivation kinetic data of MS2 for NoV inactivation by ClO_2.

The inactivation curves of NoV and MS2 in buffered media and mussel matrices from this study showed tailing phenomena. Further investigation of this phenomenon is important since its occurrence might indicate incomplete inactivation of the targeted microorganisms (Sigstam *et al.*, 2014). The tailing phenomena of inactivation curves could be explained by the consumption of ClO_2 over exposure time, as suggested by other studies (Lim *et al.*, 2010; Sigstam *et al.*, 2014). This condition was particularly supported by the ClO_2 decay rate observed from the current study, which showed similar pattern as the inactivation curves. Another possible explanation of the tailing phenomena is the occurrence of mixed population with different susceptibilities against ClO_2. Hornstra *et al.* (2011) advised that certain attachment process to particles or different disinfectant reactions that occur during treatment might instigate the presence of subpopulation within the original MS2 population. Furthermore, viral aggregation or viral clumping in the suspension was also proposed as a condition which could lead to the tailing phenomena (Thurman & Gerba, 1988). Viral aggregation could inhibit the effect of disinfectant because the consumption of disinfectant in the outer layer of viruses which leave only smaller concentration of disinfectant to react with the viruses in the inner layer (Mattle *et al.*, 2011). Thus, viruses in the inner layer will be inactivated slower than the outer part. This aggregation is often referred as a protective mechanism of core virion against disinfectant. Besides, quantification of each single non-infectious viral particle is not possible to be done in the aggregated virus, thus the number of infectious virus appears constant (Sigstam *et al.*, 2014) and observed as tails.

Based on the evaluation of ClO_2 efficacy to inactivate NoV in the current study, this compound was able to reduce NoV in the simple (buffered media) and complex matrix (mussel). Nevertheless, the direct application of ClO_2 to reduce NoV contamination in mussel matrix might not provide sufficient reductions when the reduction objective of a treatment is set at more than 2 log_{10} reductions. Using

disinfectant solution might be sufficient to eliminate microbial contamination at the food surface, however it might be ineffective to remove the virus that have penetrated inside the food matrix (Richards, 2001), such as a natural viral contamination in shellfish through the bioaccumulation process. Therefore, it can be suggested that ClO_2 is more suitable to be used as disinfectant to reduce viral contamination in the surface of matrix, for example: in the water used for washing the food handler's hand and cleaning the processing equipment, and washing or cleaning the surface of raw shellfish.

The highest NoV reduction in this study was achieved from 40 ppm ClO_2 after 120 min treatment in buffered media and mussel matrix, with the ClO_2 residue of less than 2 ppm. This ClO_2 concentration used in this study were within the range of concentration considered (5-100 ppm) by the FAO/WHO expert meeting to rinse, wash, thaw, transport and stored fish products (FAO & WHO, 2009). Moreover, the recommended ClO_2 residue as disinfectant for these purposes by Food and Drug Administration of the United States (USFDA) (CFR 173.300) is less than 3 ppm (USFDA, 2018).

The current legislation on the shellfish sanitary program in Indonesia controlled the used of chlorine in fish processing (MMAF Indonesia, 2002), while such regulation for ClO_2 has not available yet. Therefore, results from this current study can be used as input for future assessment of ClO_2 as disinfectant in fish processing practices especially for stakeholders and government in Indonesia, since this method could be used as a control strategy in shellfish processing to prevent any potential secondary contamination of NoV.

4.5. Conclusion

ClO_2 can be used as a candidate disinfectant in the processing of fishery product. At a concentration of 40 ppm for 120 mins treatment, ClO_2 gave 3.76 ± 0.35 $\log_{10}$ reduction of NoV in buffered media but only 1.65 ± 0.23 $\log_{10}$ reduction was obtained in mussel matrix. Thus, this disinfectant is more suitable to be used as a washing or cleaning sanitizer which could prevent secondary and cross-contamination of NoV during handling and processing.

Furthermore, MS2 was more susceptible than NoV towards chlorination, however the used of this surrogate is recommended to understand the kinetic mechanism of NoV in the inactivation studies. Future studies could be improved by using a closed reactor to control the ClO_2 concentration during treatment and by using different type of surrogates.

Chapter 5. Risk assessment of NoV GII in shellfish from Indonesian fish markets

5.1. Introduction

Genogroup I and II of NoV are known as human NoV and are food contaminants that can cause human gastroenteritis (Lees, 2000; Scallan *et al.*, 2011; Torok, 2013). NoV are the etiologic agents responsible for 68% of acute gastroenteritis outbreaks from 1999 to 2010 in the US (Hall *et al.*, 2013). Verhoef *et al.* (2015) studied worldwide infections due to NoV from 1999 to 2012, and reported that almost 14% of all the outbreaks are associated with food as a primary source of exposure (with other common sources including sewage contamination and exposure in child care centres, aged care homes, and cruise ships). An epidemiological study of gastroenteritis outbreaks in Europe from 1995 to 2000 (Lopman *et al.*, 2003) found that NoV, especially NoV genogroup II (NoV GII) was the major causative agent of all non-bacterial outbreaks of human gastroenteritis. Such studies demonstrate that NoV is an important source of human gastroenteritis outbreaks in many countries.

In general, enteric viral contamination of foods occurs *via* the following routes of transmission: direct contamination from human sewage and faeces/fomites, indirect contamination from infected food handlers (also known as person-to-person contamination) and through zoonotic transmission which involve animals (FAO & WHO, 2008; Verhoef *et al.*, 2015). To elaborate, following an outbreak, the infectious viral particles that were shed in the faeces or vomit of the infected person can be transmitted back to the environment, especially in the water (Montazeri *et al.*, 2015). These suspended viral particles can remain in the water for several days to weeks while maintaining the same level of infectivity (Brake *et al.*, 2018). Therefore, aquatic organisms such as shellfish, which tend to remain in the same contaminated water and filter the water to obtain food, are likely to be the most susceptible to accumulation of viruses (Lees, 2000; Montazeri *et al.*, 2015).

The presence of NoV in shellfish from different markets, restaurants and harvesting areas in Asia (Kittigul *et al.*, 2016; Maekawa *et al.*, 2007; Umesha *et al.*, 2008), Europe (Boxman *et al.*, 2006; Croci *et al.*, 2007; Le Guyader *et al.*, 2009; Li *et al.*, 2014; Loutreul *et al.*, 2014; Lowther *et al.*, 2010; Lowther *et al.*, 2012; Mesquita *et al.*, 2011; Terio *et al.*, 2010), the USA (Montazeri *et al.*, 2015), and Australia (Brake *et al.*, 2014; Symes *et al.*, 2007) has been reported. The presence of NoV in shellfish has also been directly related to gastroenteritis outbreaks (Huppatz *et al.*, 2008; Symes *et al.*, 2007). These reports emphasise the need to develop Quantitative Microbiological Risk Assessments (QMRA) for NoV as a valuable tool to estimate, and optimally manage, human health risks associated with the consumption of NoV-contaminated shellfish.

Shellfish are one of the most commonly consumed fisheries products in Indonesia. Generally, shellfish in Indonesia are consumed in cooked condition such as boiled, steamed or stir-fried and are mussels, clams or cockles. The Indonesian government recommends that shellfish in markets are cooked to open the shell, before sale to consumers (BSN, 2009). When Indonesian consumers buy raw (un-cooked) shellfish from the market, the pre-cooking step to open the shell is commonly done at home before they cook the shellfish.(Anonymous, 2018) The consumption of raw shellfish such as oysters has not yet become popular in Indonesia. In 2013, mollusc (including shellfish) production in Indonesia reached 60,471 tonnes per year, with 23,611 tonnes are intended for domestic consumption (FAO, 2015), while the major commodities being Green Mussels (*Perna viridis*), Clams (*Meretrix* spp.) and Cockle (*Anadara* spp.) (Directorate General of Fisheries, 1999; Murdinah, 2009; Setyono, 2007; WWF-Indonesia, 2015). These shellfish were mainly produced from growing areas in fresh, brackish and marine water (Nurdjana, 2006). Some of the farming and harvesting sites are located in bays and coastal waters close to human settlements, such as in Jakarta Bay, Lampung Bay (Ali *et al.*, 2015; Ferdinan, 2017; Noor, 2014; Sulvina, 2018) and Brebes (Prasetya *et al.*, 2010; Rejeki *et al.*, 2016). As a result, some growing areas might be exposed to domestic sewage including faecal pollution and therefore vulnerable to NoV contamination. Hence, there is a need to assess the risk, *e.g.,* the potential number of gastroenteritis cases due to the consumption of contaminated-shellfish

by enteric viruses (especially NoV) from Indonesian fish markets to determine risk management needs and options.

To ensure the quality and safety of shellfish harvested from growing areas, the Indonesian government initiated a program in 2004 to undertake routine monitoring and surveillance of water and shellfish quality in some shellfish growing areas (MMAF, 2004). Following this regulation, the Indonesian government also issued a standard for processing for frozen (peeled) shellfish (SNI 3460-2009) which consists of 11 handling steps (including the pre-cooking step). The pre-cooking step is defined as a method to open the shellfish shell by placing the shellfish in boiling water until the shells open and then cooling it immediately in clean water at maximum temperature of 5°C (BSN, 2009).

Although the microbiological quality of the water and shellfish are monitored, only faecal coliforms are used as a faecal pollution indicator. No tests for enteric viruses are performed during this routine monitoring. Therefore, information and data on enteric virus contamination (especially NoV) in shellfish from Indonesian fish markets is unavailable. It is, thus, difficult to estimate the NoV risk associated with shellfish consumption in Indonesia.

This study aimed to provide NoV prevalence data in shellfish obtained from Indonesian markets, especially from Jakarta and Panimbang fish markets, and to develop a risk assessment for human consumers from NoV in this commodity. This information can be used by the relevant competent authority in Indonesia to develop regulations to ensure the safety and quality of shellfish in Indonesian markets.

5.2. Materials and methods

5.2.1. Sample collection from Indonesian fish markets in Jakarta and Panimbang.

Shellfish samples were collected from fish markets in two different cities, *i.e.* Jakarta and Panimbang. In Jakarta, shellfish were purchased from two “traditional” fish markets (Cilincing and Muara Kamal) and one “modern” fish market (Everfresh), as shown in the map in Figure 5-1. In

Panimbang, shellfish were purchased from one traditional fish market. The term “modern market” describes a hygienic fish market that follows the standard sanitation practices as defined by regulations of the Indonesian Ministry of Marine Affairs and Fisheries (MMAF) (MMAF, 2017), while the term “traditional” market describes a fish market that has not applied those standard hygienic practices yet.

All shellfish purchased from traditional markets in Jakarta were harvested from Jakarta Bay, while shellfish from Panimbang fish market were harvested locally from Panimbang and Labuan (Banten Bay). However, samples from Everfresh fish market were supplied domestically and harvested from other local farming sites in Indonesia (apart from Jakarta, Panimbang and Labuan).

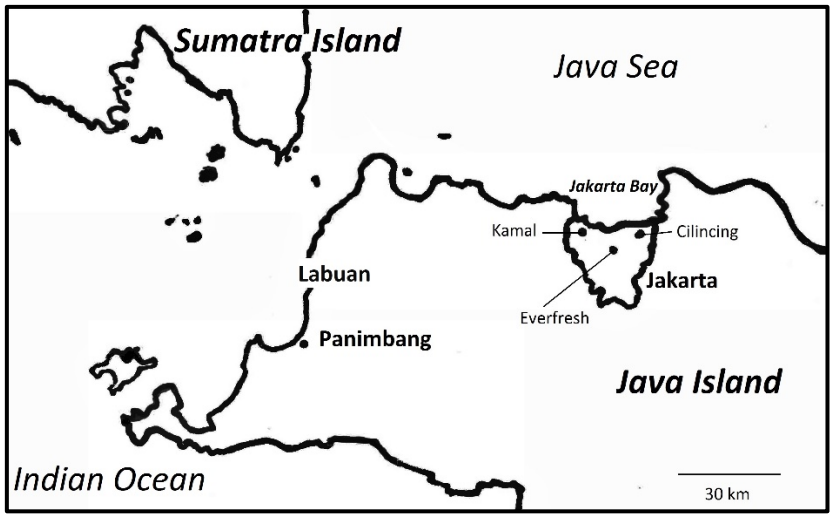


Figure 5-1. Shellfish sampling locations in Jakarta and Panimbang

Triplicate individual samples of each shellfish species from each market were collected at three times within three weeks in July 2016 and 2017. The DT were aseptically removed from the shellfish samples and stored at -20°C. Viral particles were concentrated using PEG and pre-treated using RNase enzyme as detailed in Section 5.2.2, below. The ribonucleic acids of the viruses were extracted using Trizol (Invitrogen, USA) combined with the spin column method (Yaffe *et al.*, 2012) with modifications (described in Section 5.2.2), and preserved using 70% ethanol and transported to

the University of Tasmania within 2-3 days for further purification. The samples were kept at -20°C during transportation.

5.2.2. Viral extraction and purification from shellfish digestive tissues

Viral particles were concentrated following protocols modified from Lewis and Metcalf (1988); Mullendore *et al.* (2001). Briefly, two grams of shellfish DT were inoculated with 100 µl of approximately 10^8 PFU/ml MS2 (as a process control to determine viral extraction efficacy) and homogenized in a Waring blender for 30 s at high speed with 1:4 (wt/vol) 10% tryptose phosphate broth (TPB) in 0.05 M glycine (pH 9.0). The suspension was then shaken at 250 rpm for 30 min at 4°C, and centrifuged at 5,000 x *g* for 5 min. The remaining DT were collected and stored at -20°C for further viral re-extraction (if the viral extraction efficiency of the sample was less than 10%). The subsequent concentration steps were performed as previously described (Section 3.2.6) except that for the final step of viral purification the pellet was re-suspended in 200 µl PBS, pH 7.5.

5.2.3. Plaque assay method to determine viral extraction efficiency

A hundred µl of the virus sample was analysed using plaque assay as previously described (Section 2.2.2) to determine the viral extraction efficiency. The viral extraction efficiency can be calculated as the percentage of the number of MS2 after extraction divided by total added MS2 to the sample before extraction. Following the approach of Le Guyader *et al.* (2009) only virus samples with a viral (MS2) extraction efficiency more than 10% were used for further enzymatic pre-treatment and RNA extraction as described in Section 5.2.4, below. Any virus sample with less than 10% extraction efficacy was re-extracted following the previous procedure (Section 5.2.2).

5.2.4. RNase pre-treatment and RNA extraction

The viral suspension was subjected to RNase pre-treatment as previously described (Section 2.2.4). The ribonucleic acid was extracted by guanidine-phenol-chloroform (Chomczynski & Sacchi, 2006) followed by the spin column method (Yaffe *et al.*, 2012) with modifications, as follows. In brief, 100 µl viral suspensions isolated from shellfish samples were mixed with 1 ml Trizol reagent in 1.5 ml

microtubes. Two hundred μl of chloroform:isoamylalcohol (24:1 v/v) was then added to the sample and mixed up and down for 15 sec. The suspension was centrifuged at 12,000 x *g* for 10 min at 4°C and the aqueous phase was then transferred to new microtubes containing 500 µl isopropanol and 10 µl of 1mg/ml glycogen (Sigma Aldrich, USA). This sample was incubated for 2 hours at -20 °C and then centrifuged 12,000 x *g* for 10 min at 4°C. The supernatant was discarded, and the pellet was dissolved in 350 μl GuSHCl buffer. The suspension was then added to an equal volume of 70% ethanol, mixed well and stored at -20°C. In subsequent extraction steps, the mixture was transferred to a spin column (Qiagen, Germany) and centrifuged at 8,000 x *g* for 30 s at 4°C. The eluate was discarded, and the column was washed three times: once with 500 µl 3 M Na-acetate and then twice with 500 µl 70% ethanol to remove salts. Between and after washes, the column was centrifuged at 8,000 x *g* for 30 s at 4°C and the eluate collected and discarded. The column was 'dried' by centrifugation at 7,000 x *g* for 2 min at 4°C. For elution of the RNA from the column, 50 μl of DEPC-treated water at 60°C were added directly to the column membrane, incubated for 2 min at room temperature and centrifuged at 8,000 x *g* for 2 min at 4°C. The eluate, containing the nucleic acid, was kept and stored at -70°C.

5.2.5. Enumeration of NoV by RT-qPCR

Due to unavailability of GI standard plasmid in Tasmanian Institute of Agriculture (TIA) laboratory, only NoV GII assay was performed using RT-qPCR protocols previously described (Section 3.2.6.3) and was done in duplicate per sample as confirmation step to avoid a false positive and negative result. The negative result is defined as a sample with NoV concentration below the LOD value. Only the highest NoV concentration from each positive sample was used for further study. The LOD and LOQ of this assay were determined following MIQE guidelines for real-time PCR assay (Bustin *et al.*, 2009) and suggestion by Forootan *et al.* (2017).

5.2.6. Statistical analysis

A chi-squared test was used to analyse whether the different sources and species influenced the amount of NoV contamination in the shellfish, while Analysis of Variance (ANOVA) and the Duncan Test were used to assess the significance of differences in NoV contamination level among species. These calculations were performed using Microsoft Excel and Real Statistics Resource Pack add-in, and SigmaPlot ver.12.5 (Systat Software Inc., UK).

5.2.7. Genotyping

The genotype of all NoV GII (*i.e.,* samples > LOD) detected from the shellfish samples was determined by sequencing using a CEQ^{TM} 8000 Genetic Analysis (Beckman Coulter System, USA) at the Molecular Laboratory of the Central Science Laboratory, University of Tasmania. Sequences of NoV GII ORF1-ORF2 junction region were amplified by RT-nested PCR as previously described by Kageyama *et al.* (2003) using G2FB and G2SKR as forward and reverse primers, respectively. The alternative primer sequences in this study, *i.e.*, NOV-G2-BP-F (5'-GCC CCA ATC ATG AAG ACC CA-3') as forward and NOV-G2-BP-R (5'-CAC CTG GAG CGT TTC TAG GG-3') as reverse primers, were designed using Primer-BLAST NCBI that amplify 475 bps sequence of RdRp and capsid genes (nt sequence from 4,830 to 5,304 bps which cover ORF 1 region, ORF1-ORF2 junction and ORF 2 region). These primers used when the PCR product could not be amplified using Kageyama's method due to primer mismatch with the RNA template especially in ORF1 region. The PCR products from the gel electrophoresis were purified using a QIAquick Gel Extraction Kit (Qiagen, Germany) and sequenced using GenomeLab DTCS – Quick Kit (Beckman Coulter, USA) according to the manufacturer's instructions. Sequences were analysed and corrected using BioEdit Alignment Editor (Hall, 1999). The sequences of PCR products were aligned with the published sequences from Gen Bank database using the NCBI-BLAST (Basic Local Alignment Search Tool) available at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Phylogenetic analysis was performed using MEGA 6 software (Tamura *et al.*, 2013).

5.2.8. Quantitative risk assessment of NoV in shellfish from Indonesian markets

A risk assessment (RA) was performed following the principles and guidelines for microbiological risk assessment (MRA) established by the Codex Alimentarius Commission (CAC), including a structural approach that consists of hazard identification, hazard characterization, exposure assessment and risk characterisation (FAO & WHO, 2001). A point-estimate model that determining some "worst-case" scenarios was used to develop the risk assessment. This model employs a single number of each data set which is used as an input in the risk calculation (Lammerding & McKellar, 2004; Zwietering & Nauta, 2007), for example mean of prevalence, highest level of contamination or average of shellfish consumption. This deterministic approach is a suitable model for developing the quantitative risk assessment of NoV in shellfish from Indonesian fish markets due to the paucity of some Indonesian data inputs such as NoV outbreak cases, incidences of NoV illness associated with shellfish consumption, and the proportion of shellfish species consumed by the Indonesian population. The data for NoV inactivation by thermal inactivation (Chapter 3 of this thesis) was used to calculate the potential NoV reduction after handling and cooking of the commodities.

The current regulation concerning processing of frozen shellfish in Indonesia (SNI 3460-2009) requires boiling the shellfish in the boiled water until the shells open (assumed as heating at 90-100°C for approximately 3-4 min) (Hewitt & Greening, 2006)), before the shellfish can be sold in the market (BSN, 2009). This pre-cooking method is considered in this study, and its effectiveness to reduce the risk of illness is estimated and compared with the non-pre-cooking method.

To estimate the risk of NoV, the dose per shellfish serving for different marketed shellfish (pre-cooked or non-pre-cooked) and various formats of shellfish consumed (*i.e.*, boiled, steamed and stir-fried) per consumer were calculated using Equation 5-1 and 5-2. These equations were developed in this study based on the combination of previous dose equations from Tenuis and colleagues (1997) and Pintó and colleagues (2009), and were adjusted with the variety of assumptions and the worst-case scenarios used in this study (for details see Table 5-1). The calculated dose was then used to estimate the probability of illness (P^*_{ill}) per consumer according to the exponential model as

previously described by Teunis *et al.* (1997) (Equation 5-3). The estimated number of NoV cases (*N*) based on the marketed shellfish formats (*i.e.*, pre-cooked and non-pre-cooked) and the specific cooking method of consumed shellfish (*i.e.*, boiled (N_b), steamed (N_s) or stir-fried (N_f)) per year was then calculated using the equation developed in this study (Equation 5-4). Moroever, the number of total NoV cases per year due to the assumption of 'mixed' cooking methods (N_M), can be calculated as the sum of N_b, N_s *and* N_f ($N_M = N_b + N_s + N_f$). The mixed cooking method was assumed as the combination of boiling, steaming and stir-frying method in equal proportion used to cook the shellfish by Indonesian consumer. Parameters involved in these equations are detailed in Table 5-1. The NoV dose per serving when pre-cooking and non-pre-cooking step was applied to the marketed shellfish is also calculated using Equation 5-1 and 5-2, respectively.

$$Dose = P \times C \times p \times 1/R \times I \times 10^{-\left(pre+Log\left(N/N_0\right)\right)} \times W \quad \text{(Equation 5-1)}$$

$$Dose = P \times C \times p \times 1/R \times I \times 10^{-\left(Log\left(N/N_0\right)\right)} \times W \quad \text{(Equation 5-2)}$$

$$P*_{ill} = 1 - e^{-r \times Dose} \quad \text{(Equation 5-3)}$$

$$N = E_P \times CM \times P*_{ill} \quad \text{(Equation 5-4)}$$

Table 5-1. The parameter utilised in the risk assessment to estimate the dose per serving, the probability of illness and the number of NoV cases per year

Parameter	Description	Unit	Reference	Note
P	The prevalence of NoV in shellfish from Indonesian fish markets	%	This study	The average prevalence (as worst-case scenario)
C	The highest NoV concentration in the contaminated DT shellfish	copies/g DT	This study	The maximum NoV concentration (as worst-case scenario)
p	The proportion of DT from the total weight of shellfish tissue	%	(Grodzki *et al.*, 2014)	The maximum proportion (as worst-case scenario)
R	The average recovery of the extraction method	%	This study	The minimum value (as worst-case scenario)
I	Proportion of infective viral particles among the detected viruses	%	This study	The maximum proportion (as worst-case scenario)
pre	The viral log reduction due to the pre-cooking step	Log_{10} reductions	(Hewitt & Greening, 2006)	The minimum value of viral log_{10} reductions (as worst-case scenario)
$Log(N/N_0)$	The log reduction of NoV by thermal inactivation processes that mimic the food processing styles (*i.e.*, boiling, steaming and stir-frying)	Log_{10} reductions	Chapter 3 of this thesis	$Log(N/N_0)$ of NoV at 60, 72 and 90°C treatment was applied
W	The average of shellfish consumption portion per consumer	gram	(Makmur *et al.*, 2014)	
r	The dose response of NoV illness	viral particle or genomic copies	(Teunis *et al.*, 2008)	
Pop	The total population of Indonesia of the year	people	(BPS-Statistics Indonesia, 2018)	
SC	The average of shellfish consumption per capita in Indonesia of the year	gram	(BPS-Statistics Indonesia, 2018; FAO, 2015)	
Con	The annual of total shellfish consumed in Indonesia	gram	This study	(*Con=Pop* x *SC*)
S	The expected (potential) contaminated servings	servings	This study	(*S*= (*P* x *Con*)/*W*)
CM	The proportion of shellfish consumption based on the consumption format	%	This study	Assumption – no relevant data available

5.3. Results

5.3.1. NoV exposure from shellfish from Indonesian fish markets

Ninety shellfish samples including Green Mussel, Blood Cockle and Oriental Hard Clam (Figure 5-2), were collected in 2016 and a further 81 samples were collected in 2017, from four different Indonesian fish markets in Jakarta and Panimbang, *i.e.*, Cilincing, Kamal, Everfresh and Panimbang market. Some species, *e.g.,* Oriental Hard Clam and Green Mussel, were not available for sampling in Everfresh and Panimbang market as detailed in Table 5-2.

Table 5-2. The numbers of shellfish samples from Jakarta and Panimbang fish markets in 2016 and 2017

Sampling site	Market		Year	Σ samples (per species)			Σ samples (per site per year)
	Name	Type		Oriental Hard Clam	Blood Cockle	Green Mussel	
Jakarta	Cilincing	Traditional	2016	9	9	9	27
			2017	9	9	9	27
	Kamal	Traditional	2016	9	9	9	27
			2017	9	9	9	27
	Everfresh	Modern	2016	n/a	9	9	18
			2017	n/a	9	9	18
Panimbang	Panimbang	Traditional	2016	6	6	6	18
			2017	n/a	9	n/a	9
Total				42	69	60	171

**Note: n/a =not available in the market at the time of sampling*

Green Mussels (*Perna viridis*)

Blood cockles (*Anadara granosa*)

Oriental Hard Clams (*Meretrix lusiora*)

Figure 5-2. Shellfish species collected from Indonesian fish markets

5.3.1.1. The efficiency of virus extraction and RNase pre-treatment process

To evaluate the efficiency of the virus extraction process from DT samples, MS2 was added as a process control. The average extraction efficiency was analysed by comparing the calculated number of MS2 (PFU/g) in the virus samples added before and those enumerated after the viral extraction (% recovery). Following each market sampling in 2016 and 2017 (Section 5.2.1), three individual samples from the approximately 30 samples were randomly picked and analysed by plaque assay to evaluate the efficiency of viral extraction. The average efficiency of this extraction procedure and RNase pre-treatment varied between 17.70 and 30.35% per batch (Table 5-3).

Table 5-3. The average extraction efficiency of MS2 as a control per batch

Batch No.	Week	Year	Recovery (%) Average
1	1	2016	30.35 ± 17.70
2	2	2016	31.06 ± 15.53
3	3	2016	27.01 ± 11.50
4	1	2017	21.87 ± 10.28
5	2	2017	17.70 ± 9.18
6	3	2017	24.33 ± 9.53

5.3.1.2. NoV prevalence and enumeration in the shellfish from Indonesian fish markets

Positive samples were defined as shellfish that were contaminated with NoV at a concentration above the limit of detection (LOD) (10 copies/g or 1 $\log_{10}$ copies/g DT) with no non-specific amplification products (as determined by melt curves analysis in the RT-qPCR). The average proportion of positive DT in Blood Cockles, Oriental Hard Clams and Green Mussels varied between 5 to 10% of total tissue weight (data not shown). The average NoV prevalence in shellfish from 2016 and 2017 sampling periods was 5.55% and 7.41%, respectively. The highest prevalence of NoV GII was found in Green Mussels (10%), followed by Oriental Hard Clams (7.14%) and Blood Cockles (2.9%) (Table 5-4), and all of the positive samples (>LOD) originated from "traditional" fish markets in Jakarta (Table 5-5). No positive samples were detected in the Clam samples in 2016, but 3 positive samples were found in 2017. The chi-square analysis showed that the NoV prevalence between shellfish species was not significantly different (P>0.05) (Table 5-4) but was significantly different between market sources (P<0.05) (Table 5-5).

Table 5-4. NoV prevalence in the shellfish samples from Indonesian fish markets according to species

Species	Year (Positive/Total Samples)		Total	Prevalence (%)
	2016	2017		
Oriental Hard Clam (*Meretrix lusoria*)	0/24	3/18	3/42	7.14[a]
Blood Cockle (*Anadara granosa*)	1/33	1/36	2/69	2.90[a]
Green Mussel (*Perna viridis*)	4/33	2/27	6/60	10[a]

**The same letter in the same column denotes no significant differences (P>0.05)*

Table 5-5. NoV prevalence in the shellfish samples from Indonesian fish markets according to sampling sites

Sampling sites	Market type	Year (Positive/Total Samples)		Total	Prevalence (%)
		2016	2017		
Jakarta	Traditional	5/54	6/54	11/108	10.19[a]
	Modern	0/18	0/18	0/36	0[b]
Panimbang	Traditional	0/18	0/9	0/27	0[b]

**The same letter in the same column denotes no significant differences (P>0.05)*

The concentrations of NoV (GII) in the contaminated shellfish collected in 2016 and 2017 are presented in Table 5-6. The LOQ of this assay is 20 copies/g or 1.3 log_{10} copies/g DT. The level of NoV contamination in Oriental Hard Clam species was higher and significantly different ($P<0.05$) to Blood Cockle and Green Mussels, however, there was no significant difference ($P>0.05$) between cockles and mussels.

Table 5-6. NoV concentration in contaminated shellfish at traditional markets in Jakarta according to species

Species	**NoV (log_{10} copies/g DT)**	
	For each positive sample	**Average**
Oriental Hard Clam (*Meretrix lusoria*)	2.71 2.78 3.95	3.14±0.70[a]
Blood Cockle (*Anadara granosa*)	1.60 2.18	1.89±0.41[b]
Green Mussel (*Perna viridis*)	1.43 1.54 1.92 2.48 2.48 2.57	2.07±0.51[b]

**LOQ of RT-qPCR is 1.30 log_{10} copies/g sample.*
The same letters in the same column denotes no significant differences (P>0.05)

5.3.2. Genotyping of NoV GII isolated from contaminated shellfish

A total of eleven NoV-positive samples were analysed for genotyping study. Samples were amplified by a conventional RT PCR using G2FB and G2SKR primers following the method of Kageyama *et al.* (2003) to produce 479 bps fragment. Only one of 11 samples was successfully amplified (with cDNA concentration of <25 ng/µl), and was later identified as genotype GII.4 (sample C2C3) (Figure 5-3). The alternative primers designed in this study were used to amplify the fragment from the ORF1, ORF1-ORF2 junction and ORF3 regions from the remaining positive samples that could not be

amplified by Kageyama's method. Using these primers, another sample (K3C2) produced a 475 bp fragment (with cDNA concentration of <25 ng/µl) and was also identified as genotype GII.4 (Figure 5-3). In both genotyping processes, instead of applying cloning step, two primers from each PCR method (Kageyama's and alternative method) were used in the sequencing process to avoid noisy area or poor sequences resolution due to a possible mixture of RNA from other GII strains in the sample.

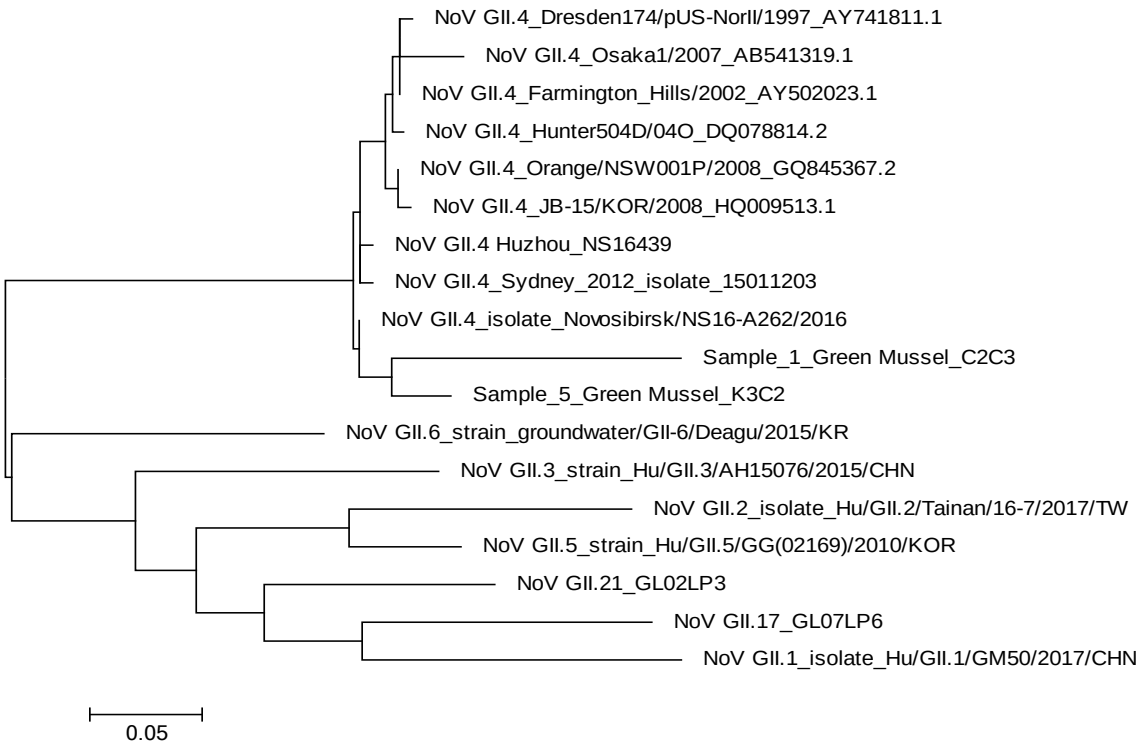


Figure 5-3. Phylogenetic tree of NoV GII detected from contaminated samples of Indonesian shellfish

5.4. Discussion

5.4.1. Prevalence and contamination levels of NoV in shellfish from Indonesian fish markets

This study presents the first data of NoV GII prevalence and contamination levels in some shellfish species commonly purchased from Indonesian fish markets in Jakarta and Panimbang for human consumption. The sampling sites were selected based on the market types (modern and traditional) as well as the source of the marketed shellfish.

Shellfish from three traditional markets in Jakarta included shellfish harvested from more polluted environments (Jakarta Bay), while shellfish from Panimbang (Banten Bay) market represented shellfish from less polluted areas. As shown from the results of this study, the highest NoV prevalence was found in Green Mussels from traditional markets in Jakarta. NoV was also found in other types of shellfish from these markets but was not found in shellfish from Panimbang market, which are mainly procured from Banten Bay. Banten Bay has been proposed by the Indonesian MMAF as a potentially safer alternative to the shellfish growing sites in Jakarta Bay (Andriyanto, 2018) which are heavily exposed to industrial and domestic sewage (Dsikowitzky *et al.*, 2016; Siregar *et al.*, 2016).

NoV GII.4 were identified from Green Mussels in this study. Genogroup GII, and especially GII.4, are the most common cause of human gastroenteritis outbreaks (Bernard *et al.*, 2014; Bull *et al.*, 2006; White, 2014). Thus, these findings emphasize the importance of regular monitoring and surveillance of NoV in shellfish products. Although regular monitoring and surveillance is carried out by local and national Indonesian authorities to ensure the safety and quality of shellfish in Indonesian markets, information on NoV prevalence remains limited. The Indonesian government has mainly focused on monitoring of biotoxins, heavy metals and bacterial contamination in shellfish products (MMAF, 2004), in which only the faecal coliform test was used as an indicator of faecal contamination in the growing areas (BPLHD, 2015). If the results of routine monitoring indicate high levels of faecal coliform contamination (> 300 MPN faecal coliforms/100 ml) in the water, and further laboratory tests confirm these observations, the authorized agency is required to perform an evaluation of the particular area and, as appropriate, the area will be declared as “off-limits” and no shellfish growing and harvesting can be done in this area until the water quality improves and meets the requirements (MMAF, 2004).

The use of faecal coliforms as an indicator for faecal contamination may not an effective approach to assess NoV contamination of shellfish or harvesting sites. While the faecal coliform test can effectively indicate enteric bacterial pathogens in shellfish and its production areas (Suffredini *et al.*,

2014), it is less accurate to assess enteric virus contamination in the shellfish or viral dispersal in sewage-contaminated water (Brake *et al.*, 2018; Lee *et al.*, 2013; Winterbourn *et al.*, 2016).

5.4.2. Quantitative Risk Assessment of NoV in Shellfish from Indonesian markets

A Quantitative Microbiological Food Safety Risk Assessment (QMFSRA) utilising the NoV prevalence and contamination levels data from this study was performed with different assumptions, including 'worst-case scenarios', (as described in Table 5-1) to provide scientific information on the risk of NoV infections due to the consumption of shellfish in Indonesia. The QMFSRA was performed using a deterministic approach following the guidance and example of the risk evaluation of viruses in oysters in UK (ICMSF, 2018) and a model of HAV in shellfish (Pintó *et al.*, 2009), with modifications to the input parameters used for the risk calculations. The risk assessment in this study employed various assumptions such as the application of a pre-cooking step to raw shellfish and also considered the consumer behaviour toward shellfish cooking and consumption in Indonesia, where the shellfish are usually consumed in the cooked form, *i.e.*, after being boiled, stir-fried or steamed. These pre-cooking and cooking practises could potentially eliminate or greatly reduce the possibilities of NoV contamination in the final shellfish product. Hence the data on NoV thermal inactivation (which mimics those cooking processes) from the Chapter 3 of this thesis were integrated into the risk characterization.

5.4.2.1. Hazard identification

Occurrences of NoV in food as well as reported cases of NoV infection due to the consumption of contaminated food in some developed countries have been well-reported (Lopman *et al.*, 2003; Scallan *et al.*, 2011). In some developing countries such as Indonesia, however, foodborne outbreaks caused by NoV are undocumented or underreported. The available published data on NoV infections in some developing countries are limited to NoV prevalence from symptomatic and asymptomatic patients such as in African (Armah *et al.*, 2006; Ayukekbong *et al.*, 2014), South American (Bucardo *et al.*, 2017; Fumian *et al.*, 2016; García *et al.*, 2006), and Asian countries

including Indonesia (Nguyen *et al.*, 2007; Sai *et al.*, 2013; Subekti *et al.*, 2002a; Subekti *et al.*, 2002b; Utsumi *et al.*, 2017). In Indonesia, prevalence of NoV in patient stool samples varied between 2.7 to 20.6% (Subekti *et al.*, 2002a; Utsumi *et al.*, 2017). Although some NoV infections were recorded from the patient, the information about the source of these infections were not available.

According to FAO data, mollusc (including shellfish) production in Indonesia increased from 53,684 to 60,471 metric tonnes in the period of 2010 to 2013 (FAO, 2015). However, some of the shellfish growing and harvesting areas in Indonesia, such as Jakarta Bay, are located close to estuaries and likely to be contaminated by domestic sewage from the surrounding settlement (Dsikowitzky *et al.*, 2016). As described earlier (Section 1.1.5), shellfish are highly susceptible to microbial contamination, including viruses, due to their filter feeding behaviour (Le Guyader *et al.*, 2013; Lees, 2000). Since NoV are highly persistent in the water environment (Brake *et al.*, 2018; Cook *et al.*, 2016), shellfish grown in NoV contaminated areas are at high risk of being contaminated by NoV.

Current Indonesian national standards for shellfish products sold in the market provide guidelines on safety and quality requirements as well as handling and processing of frozen (SNI 3460.1 to 3: 2009) and canned shellfish (SNI 3919.1-3:2009). However, related regulations for fresh shellfish do not exist, although this product is often sold fresh to consumers. Moreover, these regulations do not consider viruses as potential microbial contaminants for raw material intended for frozen and canned products.

5.4.2.2. Exposure assessment

Section 5.3.2. detailed the NoV prevalence from shellfish sold in Indonesian markets. A total of 171 samples were collected from four markets in Jakarta (Special Capital Region of Jakarta Province) and Panimbang (West Java Province) in 2016 and 2017 (Table 5-2). As presented in Table 5-4 and Table 5-5, the average NoV prevalence was 6.48% and all positive shellfish were originated from traditional markets in Jakarta. The shellfish sold in these markets are more likely to be grown in, or harvested from, Jakarta Bay which has experienced environmental stress due to high loads of solid

waste and wastewater from the surrounding households and industries from the city of Jakarta (Dsikowitzky *et al.*, 2016). Most of the wastewater is only partially treated or untreated and collects into 13 rivers and canals which empty into Jakarta Bay (Nur *et al.*, 2001).

Shellfish consumption data were estimated from the data of FAOSTAT (FAO, 2015) and the Indonesian Ministry of Health (Indonesian Ministry of Health, 2014). FAOSTAT estimated that the total domestic supply of molluscs intended for consumption in Indonesia in 2013 was 23,611 tonnes (FAO, 2015). Furthermore, the national food consumption survey in Indonesia in 2014 suggested that the number of consumer consuming squid and shellfish per year was 1.1% of the total population (Indonesian Ministry of Health, 2014). This survey used a cross sectional design and was conducted in every province in Indonesia. Consumer food intake during the last 24 h was recorded from 191,524 participants from 51,127 households.

The average amount of shellfish consumed per serving is estimated as 185.29 g, derived from a study in Cilincing, North Jakarta (Makmur *et al.*, 2014). This survey involved 200 participants with inclusion criteria as those who consume shellfish.

In the current study, due to unavailability of recorded or published data on the consumption of raw shellfish in Indonesia, the proportion of shellfish consumed by Indonesian consumer was estimated based on assumptions of different shellfish cooking methods. The most common cooking practices of shellfish in Indonesia are boiling, steaming and stir-frying (Murdinah, 2009; Panjaitan *et al.*, 2018; Wongso & Tobing, 2012). Because there is lack of information and data about the proportion of the different shellfish cooking methods, further assumptions were made on these proportions in this current study (Table 5-7) to assess the importance of those assumptions on the risk estimates.

Table 5-7. Assumptions on the proportion of shellfish cooked by different methods

Assumptions	Percentage of cooking methods (%)		
	Boiling (90-100°C for 30 min)	Steaming (72°C for 15 min)	Stir-frying (60°C for 30 min)
1	100	0	0
2	0	100	0
3	0	0	100
4 ("mixed")	33.3	33.3	33.3

The above proportions of cooking methods were estimated based on the antimicrobial potency of each cooking method as well as the "mixed" method (assumption 4) to reduce NoV contamination in the shellfish, thus the relative efficacy of different cooking method to reduce the risk of NoV cases can be determined.

5.4.2.3. Hazard characterisation

Since specific studies on the dose response of Indonesian consumers (patients) to NoV exposure are not yet available, the probability of infection in this study was calculated using the dose response model developed by Teunis *et al.* (2008), while the NoV concentration and the serving size estimates were provided in Table 5-6 and Section 5.4.2.2, respectively. Teunis's model was derived from the infectivity of NoV in human challenge studies, where the lD_{50} was estimated to be 1 million particles or viral copies (ICMSF, 2018). The probability of illnesses due to the consumption of NoV contaminated shellfish was estimated using a simple exponential model (Teunis *et al.*, 1997).

To coordinate the available data with the required input values in this quantitative approach, several assumptions were made. The NoV contamination level was analysed from the shellfish DT, which comprise approximately 10% (assumed as the maximum proportion) of total shellfish tissue weight (Grodzki et al., 2014). Although a previous study showed that the majority of the viral particles were accumulated in the DT and were not homogenously distributed in other shellfish organs (McLeod *et al.*, 2009), however some other studies confirmed that viral particles were not only accumulated in the shellfish DT but also in other organs such as gills, adductor muscle and haemolymph cell

(Maalouf *et al.*, 2010). Therefore, in this study the viral particles were assumed to be distributed not only in the DT but also in other organs, as a worst-case scenario. This approach was applied to avoid an underestimation of the dose calculated. The conversion factor (p) of 10%, which was obtained as the proportion of digestive tissue from the total weight of shellfish tissue was used to calculate the dose. The highest level of NoV contamination in shellfish from this study (8,980 copies/g DT before adjustment by correction factor of recovery rate (*R*)) was also chosen as the worst-case scenario in this risk assessment. Moreover, as there is no data available on the proportion of shellfish consumption based on different species of shellfish, it was assumed that the population consumed similar proportions of each shellfish species. A further assumption about the total shellfish (bivalve molluscan shellfish) consumption was also made, since the FAO data on the total domestic supply was calculated for molluscs in general (includes bivalve molluscan and other molluscs without shell). All the required data and information to calculate the risk estimate are presented in Table 5-7.

Table 5-8. Input parameters for the deterministic QRA to estimate the risk of NoV in shellfish from Indonesian fish markets

Parameter	Values	Reference(s)	Note
P	6.48%	Chapter 5 (Section 5.3.1) of this thesis	Assumed at the average of annual prevalence
C	8,980 copies/g DT	Chapter 5 (Section 5.3.1) of this thesis	Worst-cases scenario
p	10%	(Grodzki *et al.*, 2014)	Assumed at max. proportion
R	17.70%	Chapter 5 (Section 5.3.1) of this thesis	Worst-cases scenario
I	100%	Chapter 5 (Section 5.4.2) of this thesis	Worst-cases scenario
pre	2	(Hewitt & Greening, 2006)	Worst-cases scenario
$Log(N/N_0)$	4 (at 90°C for 30 min); 3 (at 72°C for 30 min); and 1 (at 60°C for 30 min)	Chapter 3 (Section 3.3.4) of this thesis	
W	185.9 g	(Makmur *et al.*, 2014)	
r	1/1,000,000 copies or viral genomic	(Teunis *et al.*, 2008); (ICMSF, 2018)	
Pop	248,800,000	(BPS-Statistics Indonesia, 2018)	
SC	94.9 g	(BPS-Statistics Indonesia, 2018; FAOSTAT, 2015)	
Con	23,611,120,000 g	-	Calculated
S	8,230,234.41 servings	-	Calculated
CM	Boiling; steaming; and stir-frying*	-	Assumed

*Note:*The details on the assumption of cooking methods proportion were described in Table 5-7*

5.4.2.4. Risk characterisation

Based on the thermal inactivation data from this thesis (Section 3.3.4) the reduction of NoV by a cooking process such as boiling at 90-100°C for 30 min, steaming at 70-80°C for 30 min or stir-frying at 60°C for 30 min were predicted to be at least 4, 3 and 1 $\log_{10}$ reductions, respectively. These log reductions values of NoV in shellfish matrix due to thermal inactivation that mimicked the assumed cooking process were estimated using a Biphasic (non-linear) model. In addition, the result from previous study showed that >2 $\log_{10}$ viral reductions were achieved by boiling until the shell opens (90°C for 3-4 min) (Hewitt & Greening, 2006). This value (2 $\log_{10}$ reductions) was used to determine the minimum viral reduction achieved by the pre-cooking step (*i.e.,* the worst-case). All of the viral reduction values were then incorporated with the NoV annual prevalence and concentration data, the average mass of shellfish consumed by the Indonesian population, the recovery rate of the quantification method, the proportion of DT from the total shellfish body weight and the proportion of virus infectivity in the sample, to estimate the doses of NoV per serve of shellfish (Equation 5-1 and 5-2).

By multiplying the estimated average probability of illnesses (P^*_{ill}) with the potential contaminated servings, the annual NoV incidences based on the various assumptions of the most common shellfish cooking methods in Indonesia with the worst-case scenarios were estimated. The results are presented in Table 5-9. The annual attack rates of NoV (number of NoV-illness cases per 100,000 inhabitants per year) due to contaminated-shellfish consumption in Indonesia are presented in Table 5-10. These attack rates depend on the assumptions in the application of pre-cooking step of pre-marketed shellfish as well as the cooking methods. For instance, when the pre-cooking step (boiling at 90-100°C for 3-4 min) was incorporated into the risk calculation, the attack rates of each cooking methods were, as expected, 100-fold lower than without a pre-cooking step (Table 5-10). It can be explained because, from the results from previous studies (Hewitt & Greening, 2006), pre-cooking by boiling at 90-100°C for-3-4 min to open the shell reduces NoV particles by at least 2 $\log_{10}$.

Table 5-9. The NoV-illness cases per year estimated based on the assumption of the most common shellfish cooking methods in Indonesia with the worst-cases scenario

Assumption on shellfish cooking method	No of cases
Without pre-cooking	
All shellfish cooked by boiling (90-100°C for 30 min)	780
All shellfish cooked by steaming (70-80°C for 30 min)	7,800
All shellfish cooked by stir-frying (60-70°C for 30 min)	741,000
Shellfish cooked by mixed method[1]	250,000
With pre-cooking[2]	
All shellfish cooked by boiling (90-100°C for 30 min)	7.8
All shellfish cooked by steaming (70-80°C for 30 min)	78
All shellfish cooked by stir-frying (60-70°C for 30 min)	7,800
Shellfish cooked by mixed method[1]	2,600

Note: [1]The mixed method was assumed as mixed cooking practices consist of boiling, steaming and stir-frying in equal proportion (33.33% of each cooking method)
[2]The standard handling procedures of pre-marketed raw or frozen peeled shellfish published by Indonesian government which utilise boiling step (boiling at 90-100°C for 3-4 mins) to open the shell

Results from previous studies by Pintó *et al.* (2009), which estimate the risk of enteric viruses in shellfish products in Spain and the documented enteric viruses outbreaks due to shellfish consumption by Suffredini *et al.* (2014) were compared to the results from this study. The estimated NoV attack rates in Indonesia assumed without pre-cooking step were higher than those reports, but when including the pre-cooking application, the rates were comparable to those estimates in Spain and Italy (Table 5-10). However, the estimated NoV attack rates in Indonesia were lower than the attack rate from the recorded HAV cases in China during the outbreaks in 1988 (Halliday et al., 1991) or from the estimated NoV cases example in UK due to raw shellfish consumption (ICMSF, 2018).

Table 5-10. The estimated and reported attack rate of enteric virus due to shellfish consumption in different scenario in one-year period

Scenario	Attack rate (per 100,000 person)	Note
This study		
No pre-cooking + boiling only	0.31	Estimated
No pre-cooking + steaming only	3.09	Estimated
No pre-cooking + stir-frying only	295.43	Estimated
No pre-cooking + mixed cooking	99.61	Estimated
Pre-cooking + boiling only	0.0031	Estimated
Pre-cooking + steaming only	0.031	Estimated
Pre-cooking + stir-frying only	3.09	Estimated
Pre-cooking + mixed cooking	1.04	Estimated
Other studies		
No cooking (Pintó *et al.*, 2009)	0.66-0.91	Estimated
Lightly cooking (Pintó *et al.*, 2009)	0.05-0.43	Estimated
Well cooking (Pintó *et al.*, 2009	0.01-0.21	Estimated
Raw consumption (in UK)*	3,000	Estimated example
High pressure process (in UK)*	3.08	Estimated example
HAV prevalence studies (in Peru) (Pintó *et al.*, 2009)	3.30-13.30	Estimated
Italia outbreaks in 2008 (Suffredini *et al.*, 2014)	2.5	Reported
China outbreaks in 1988 (Halliday *et al.*, 1991)	4,083	Reported

Note: Example of risk estimation of NoV cases in UK (ICMSF, 2018)*

5.4.2.5. Limitations of the risk assessment and future recommendations

The estimated risk of illnesses and the attack rates due to the consumption of NoV-contaminated shellfish in Indonesia were different from other outbreak estimates due to enteric viruses which used a similar risk estimation approach (ICMSF, 2018; Pintó *et al.*, 2009), especially when the pre-cooking method was not considered. These differences could be due to the various assumptions and the worst-case scenarios that were used in this current study. In the calculation of virus dose per serving, this study assumed that the proportion of DT from the total weight of shellfish tissue was 10% (Table 5-1) because the NoV concentration was calculated only from the sample's DT, while the study of Pintó *et al.* (2009) did not use this correction factor and assumed that the level of HAV contamination in the shellfish DT represented the contamination throughout the flesh of the individual shellfish. Furthermore, because of the unavailability of an *in-vitro* assay method to evaluate the infectivity of the NoV in this study, NoV quantified by RT-qPCR with enzymatic pre-treatment were assumed as infectious viral particles in the risk estimation. If an *in-culturo* assay

becomes available as a standard method to quantify the levels of infectious NoV (such as HAV quantification assay), the current risk assessment may be improved.

The worst-case scenarios used in this study were made to accommodate data gaps on shellfish consumption and preparation methods in Indonesia. Based on the prevalence study, the highest NoV contamination was found in Clams (Table 5-6). Thus, to generate the maximum risk estimate, it was assumed that all shellfish consumers in Indonesia only consume Clams. Following this assumption, the highest concentration of NoV in Clams (8,980 copies/g DT) was used in the risk calculation. In addition, the lowest recovery average was also used in the risk calculation to develop a worst-case risk estimation.

To resolve these data gaps and refine the risk estimates and potential risk management solutions, more detailed studies on the volume of different shellfish species consumed by Indonesian consumer is necessary to follow up the National Food Consumption Survey (SKMI) conducted by the Ministry of Health. In addition, to get more representative information on the NoV prevalence in shellfish from Indonesian markets, further studies or surveys should also be carried out in other Indonesian fish markets. To properly identify the origin of shellfish contamination, direct sampling of waters from the shellfish growing areas is also suggested. This could also provide information on the actual level of NoV in shellfish due to faecal-oral transmission (natural contamination).

The current risk assessment focussed on the efficacy of heat treatment as a potential control measure to reduce NoV contamination in shellfish. The pre-cooking practice, which was proposed by the Indonesian government for frozen (peeled) shellfish (SNI 3460:2009), was included as an assumption in the risk calculation. This processing step is intended to open the shellfish shell (BSN, 2009). As shown in Table 5-10, assuming that the pre-cooking step was applied with a further cooking method, the number of estimated NoV cases as well as the attack rates per year due to shellfish consumption can be reduced to 100-fold. For example, pre-cooking before boiling reduced

the estimated cases of illness due to the consumption of NoV-contaminated shellfish from 780 cases (without pre-cooking) to 7.80 cases (with pre-cooking) per year. This standard processing supports conclusions from a previous study (Hewitt & Greening, 2006) and guidelines from Codex Alimentarius Commission (CAC) on the general principles of food hygiene to control viruses in food (FAO & WHO, 2012) where boiling the shellfish at minimum 3 min resulted in increasing internal temperature of the shellfish to a minimum of 90°C, and maintaining this internal temperature for minimum 90 s was recommended to inactivate viruses in most foods.

Following the different methods of cooking that are generally done by shellfish consumers in Indonesia (Table 5-10), pre-cooked shellfish with further boiling have the lowest risk of residual NoV contamination in the final product, while the highest-risk product is estimated to be stir-fried shellfish without pre-cooking. This observation showed that although NoV was found in shellfish sold in traditional markets in Jakarta, the current processing practices of the consumer will reduce the NoV contamination in the product. Furthermore, reduction levels are dependent upon the different cooking methods. It can be suggested that consumer should pre-cook their shellfish before further cooking and that boiling is preferable to other cooking methods to reduce the level of NoV contamination. It is also suggested that pre-cooking of raw shellfish should be done in the processing facilities before the product is sold, particularly for shellfish harvested from polluted sites such as Jakarta Bay.

However, if the consumption of raw shellfish, such as oysters, becomes popular in Indonesia in the future, the risk of illnesses due to shellfish consumption might increase beyond the estimates provided in this study, especially if the shellfish are harvested from polluted sites such as Jakarta Bay. In this scenario, the pre-cooking practices and cooking methods will not be applied, thus the risk of NoV infection will need to be re-calculated but would be expected to be tens-of-times higher per serving. In the absence of cooking steps, the quality assurance of this product from farm to fork will need to be well-monitored and controlled, *e.g.*, when the shellfish growing/harvesting sites are

determined as “off-limit” by the competent authorities, following the sites’ closure, the products are not allowed to be harvested and marketed.

5.5. Conclusion

Frequent NoV contamination was observed in shellfish obtained from traditional markets in Jakarta, which most likely are harvested from Jakarta Bay. Findings from the risk assessment presented as part of this study emphasized the value of implementing pre-cooking practices by producers and consumers, to reduce the level of NoV contamination in the shellfish, thus reducing the estimated risk of illness. Furthermore, based on the set of assumptions and scenarios in this risk assessment study, different cooking methods (*i.e.,* boiling, steaming and stir-frying) affects the number of estimated the risk of NoV cases and the attack rates, with the shellfish boiled for 30 minutes having the lowest risk product of NoV contamination, and of causing illness to consumers.

Chapter 6. General discussion and conclusions

6.1. General discussion

As reviewed in Chapter 1 (Section 1.1), NoV remains the leading causative agent of viral gastroenteritis outbreaks, and subsequent health and economic losses worldwide. Most of the outbreaks are caused by person-to-person and faecal-to-oral transmission through water and environmental contamination, whereas some of the outbreaks were associated with consumption of NoV-contaminated food (Glass *et al.*, 2009; Verhoef *et al.*, 2015). NoV can be introduced into water via sewage overflows or contaminated water from the surroundings (Aw *et al.*, 2009; Rodríguez *et al.*, 2012; Wyn-Jones *et al.*, 2011; Yang *et al.*, 2012). Hence, raw or fresh food such as shellfish, produce and fruit which are grown or harvested, irrigated, handled and processed with NoV-contaminated water have become the most common source of food-borne NoV infection. Moreover, food that is prepared and handled by NoV-infected persons (both symptomatic and asymptomatic) can also contribute to the NoV infection.

Compared to other aquatic animals, shellfish are more susceptible to NoV contamination, due to their filter feeding behaviour which enables them to accumulate different types of suspended particles from their aqueous environment, including bacteria and virus (Lees, 2000). An increasing trend in shellfish consumption and production in Indonesia has been documented since the early 2000's. The source of Indonesian shellfish is mainly from domestic production by shellfish farming or wild catch (FAO, 2015). Some of the harvesting sites have been heavily contaminated with sewage overflow from the rivers, such as Jakarta Bay (Dsikowitzky *et al.*, 2016). Currently, shellfish species harvested and caught from the Jakarta Bay (*i.e.*, Green Mussel, Blood Cockle and Hard Clam) are commonly found in the traditional fish markets close to Jakarta Bay (*i.e.*, Cilincing and Muara Kamal). It is, therefore, likely that these shellfish are being contaminated by faecal sewage containing enteric viruses including NoV.

Generally, shellfish in Indonesia are cooked before consumption, however, the risk of NoV contamination might remain due to inadequate cooking of the contaminated shellfish, secondary transmission route through cross-contamination between shellfish during washing steps or contamination from an infected food handler. However, a risk assessment of NoV from shellfish in Indonesian fish market is not yet available, hence, there is a need to develop this risk assessment especially for shellfish from traditional fish markets (*i.e.*, Jakarta and Panimbang). Such a food safety risk assessment will provide the competent authorities in Indonesia (both local and central government) with information about the estimated magnitude of risk due to consumption of NoV-contaminated shellfish as well as potential control strategies for NoV foodborne cases.

The EFSA recommended investigation of NoV levels in shellfish products following several incidents of foodborne NoV illnesses related to the consumption of raw shellfish, using robust methods for NoV identification and quantification (EFSA Panel on Biological Hazards (BIOHAZ), 2012). However, these efforts remain challenging because a standard quantification assay based on the cell-culture system as a robust quantification method is currently unavailable. As a consequence, a molecular-based method such as RT-qPCR has been used as the gold standard assay for detection and quantification of NoV (ISO, 2013, 2017; Kirby & Iturriza-Gómara, 2012; Vinjé, 2015). One of the limitations of using RT-qPCR in the quantitative analysis is the inability of this method to distinguish between infectious and non-infectious viral particles (Knight *et al.*, 2012; Richards, 1999) which could lead to overestimation of NoV and the related risks and provided inaccurate information for the decision making process.

Chapter 2 of this thesis addressed the above issue by proposing enzymatic pre-treatment to improve the ability of RT-qPCR to differentiate the infectious from non-infectious viral RNA using MS2 bacteriophage (MS2) as a cultivable NoV surrogate. The results showed that the performance of RT-qPCR without enzymatic pre-treatment was comparable to the plaque assay method only for quantification of non-heated MS2 (presumed only infectious viruses were present), but was not comparable for the quantification of infectious MS2 after heat treatment where both infectious and

non-infectious viral particles were present. In addition, by comparing the result of RT-qPCR with the culture-based method (plaque assay), the application of RNase as enzymatic pre-treatment was able to reduce the overestimation of "infectious" viral particles that survive from the treatment. The ability of RNase to reduce the overestimation of the infectious viral particles can be explained by its ability to degrade the RNA from non-infectious viral particles lacking capsid protein (Brié *et al.*, 2016; Pecson *et al.*, 2009), and thus only to quantify RNA from infectious viral particles.

However, without a further step to inactivate RNase by RNasin treatment during the enzymatic pre-treatment in this study, underestimation of the infectious viral particles was observed using RT-qPCR assay. The finding of this study showed that the application of RNase followed by RNasin prior to RNA extraction were able to reduce the overestimation of infectious MS2 from heat treatment which is confirmed by the comparable results obtained from the RT-qPCR method (with enzymatic pre-treatment) compared to the plaque assay. Hence, this RT-qPCR assay with enzymatic pre-treatment (RNase followed by RNasin prior to RNA extraction) was proposed to enumerate infectious viral particles from thermal or chlorination treatments, as described in Chapters 3 and 4.

This assay (pre-treatment RT-qPCR) was also used to in the prevalence study (Chapter 5). Quantification using this assay was able to avoid over-estimation, thus provided reliable results on the level of NoV in shellfish available at retail markets in Indonesia.

Based on the available records, most NoV outbreaks are related to the consumption of raw-contaminated shellfish (Huppatz *et al.*, 2008; Lodo *et al.*, 2014; Morse *et al.*, 1986; Westrell *et al.*, 2010), however undercooked shellfish is also reported to cause illnesses (Alfano-Sobsey *et al.*, 2012; Richards, 2006). To overcome this problem, thermal inactivation has been considered as one of the most effective treatments to reduce or eliminate enteric virus contamination (Bertrand *et al.*, 2012; Richards *et al.*, 2010; Teixeira, 2015). Heating the shellfish before consumption is an acceptable approach in Indonesia, because the majority of shellfish consumers in Indonesia cook their shellfish

before eating, therefore the heat treatment is unlikely to affect their perception of organoleptic quality of the final product.

The application of heat to inactivate NoV and MS2 in buffered media and artificially contaminated mussel (*Mytilus galloprovincialis*) was evaluated in Chapter 3. The heat inactivation kinetics of the two viruses were compared using linear (first-order kinetic) and non-linear (Weibull and Biphasic) models. The heating temperatures explored, 60, 72 and 90°C for different contact times, represented the cooking processes of stir-frying, steaming and boiling, as the most common cooking practices of shellfish in Indonesia.

In general, tailing phenomena were observed in all inactivation curves of NoV and MS2 in both matrices (buffered media and mussel matrix). These findings agree with the observations from previous thermal inactivation studies (Araud *et al.*, 2016; Bozkurt *et al.*, 2013, 2014a) where there were more heat-resistant subpopulations present during viral inactivation treatments. As a consequence, the non-linear models (Weibull and Biphasic) which have lower RMSE values, performed better in the prediction of thermal inactivation kinetics of the viruses than log-linear models (first-order kinetic).

Although those non-linear models were appropriate to describe the thermal inactivation curves of NoV and MS2 for the full duration of the treatment, only the Biphasic model was able to predict the rates of NoV elimination in both matrices after an extended period. Hence, this model was used to predict two and four $\log_{10}$ reduction (2D and 4D) of NoV in both matrices. When a non-linear model is the best to describe the survival curves, the specific viral $\log_{10}$ reductions were best predicted by direct calculation of determined values (such as 2D, 3D or 4D) from the equation rather than multiplying the D values (1D) obtained from the models with the targeted $\log_{10}$ reductions (such as 2, 3 or 4) to avoid over or under-estimation. For instance, the time for 4 $\log_{10}$ reductions (4D) calculated from the equation was not equal to the value of D values (1D) multiplies by 4 because the responses were not log-linear. Thus, when a food safety objective is determined by the minimum

requirement of a specific log_{10} reductions during processing for risk elimination purposes, the use of targeted D values (*e.g.*, 2D, 3D or 4D) predicted by non-linear models could prevent the overestimation of viral inactivation in cases where a tailing phenomenon is observed.

A difference in the efficacy of thermal inactivation against NoV and MS2 was observed in this study. Overall, MS2 was more susceptible than NoV to heat treatment in both matrices (buffered media and mussel matrix) and at temperatures between 60 and 90 °C and had higher z, D, 2D and 4D values than NoV. It has been shown that the difference in viral resistance toward environmental stress between virus species or even strains is determined by capsid protein and genomes structure of the virus (Thurman & Gerba, 1988). Therefore, MS2 may not be a good candidate for NoV surrogate in thermal inactivation studies. The efficacy of thermal inactivation is also influenced by the matrix, *i.e.* both viruses were generally more resistant towards heat treatment in a complex matrix (mussel), which is potentially due to the presence of protein and fat in the mussel that protect the viral particles from heat (Bozkurt *et al.*, 2014b; Croci *et al.*, 2012).

Another source of NoV contamination in food, identified as secondary route of contamination, is cross-contamination from food handlers or other contaminated products or equipment, which may occur during harvesting, handling or processing (Bellou *et al.*, 2013; Hall *et al.*, 2012; Polo *et al.*, 2015; Rodríguez-Lázaro *et al.*, 2012). In this case, the use of disinfectants such as chlorine to prevent cross-contamination is recommended. The considered concentrations of ClO_2 to be used as disinfectant in water are between 5-100 ppm (FAO & WHO, 2009), while the USFDA recommended the level of ClO_2 residue is less than 3 ppm (CFR 173.300) (USFDA, 2018). To this end, Chapter 4 evaluated the efficacy of chlorine dioxide (ClO_2) treatment at concentrations of 10, 20 and 40 ppm at 20 ± 1°C (pH 6.9 ± 2) with a range of contact times, to inactivate NoV and MS2 in buffered media and mussel (*Mytilus galloprovincialis*) matrix. Hom, Weibull and Biphasic models were used to estimate the log_{10} reduction of viral particles over time, while a first-order kinetic model was used to calculate ClO_2 decay rate. Overall, the viral reduction curves that were generated from the number of infectious viral particles plotted against time exposure were better fitted by Hom than other models,

especially for NoV. For some MS2 treatments (20 and 40 ppm), the Biphasic model was the best to predict MS2 reduction as a function of time. From the observed data, the highest concentration of ClO_2 (40 ppm) with the longest exposure (120 min) produces the highest log_{10} reduction of the viral particles with the ClO_2 residue <2 ppm in both matrices, thus this treatment has potential to be used as disinfectant, as considered by the FAO/WHO (FAO & WHO, 2009) and to meet the minimum residue required by the USFDA (USFDA, 2018).

A matrix effect was observed in the decay of ClO_2, where the decay rate was higher in complex (mussel) than in simple matrix (buffered media), presumably due to the presence of higher loads of organic matter. As a consequence, the rate of viral reduction of NoV or MS2 was significantly higher ($P<0.05$) in buffered media than in mussel matrix from identical treatments. In this study, the decay of ClO_2 over time was presumed to be one of the causes of the tailing phenomenon in all inactivation curves predicted by the three models (Hom, Weibull and Biphasic). This assumption is in agreement with the findings from previous studies (Thurston-Enriquez *et al.*, 2003), where one of the factors that contributed to the tailing phenomenon is the decrease of disinfectant concentration over the time (Thurman & Gerba, 1988).

The results of the current study provide evidence that the efficacy of ClO_2 treatment varies between viruses. MS2 was generally more susceptible than NoV to ClO_2 treatment in both matrices as MS2 obtained a higher log_{10} reduction than NoV for the same treatments at the same matrix. This difference in viral response to disinfectant was similar to the viral response towards heat treatment, which can be explained by the difference in viral protein structures as previously described (Sigstam *et al.*, 2013; Wigginton *et al.*, 2012). Based on the observation in this thesis together with those previous studies, MS2, which is less resistant toward heat and ClO_2 treatment, may not be suitable as a NoV surrogate to generate viral particles inactivation kinetics by these treatments. Thus, the use of MS2 as surrogate in NoV inactivation studies should be performed with caution, to avoid overestimation of the treatment efficacy. However, MS2 could be a useful enteric virus surrogate to

describe general trends and mechanisms of enteric viral inactivation studies, especially using heat and ClO_2 treatments.

As part of this study, a prevalence study of NoV in shellfish from Indonesian markets was conducted in 2016 and 2017 (Chapter 5). The aims of this study were to specifically investigate the presence of NoV and its level in the shellfish sold in two different places (Jakarta and Panimbang) and two different market types ("traditional" and "modern") to indicate the source of shellfish from less or more polluted areas.

All NoV-contaminated shellfish observed in this study were collected from traditional markets in Jakarta (Cilincing and Muara Kamal) that represented shellfish harvested from a polluted environment (Jakarta Bay). The level of NoV concentration in the contaminated shellfish varied between 1.43 to 3.95 $\log_{10}$ copies/g DT. By adjusting these values with the lowest average of acceptable viral extraction efficiency (17.7%), the estimated NoV concentration were between 2.20 to 4.72 $\log_{10}$ copies/g DT. Furthermore, no NoV were detected in shellfish collected from Panimbang fish market or from a modern market from Jakarta. Amongst different shellfish species, the highest NoV prevalence was found in Green Mussel (*Perna viridis*). This finding might be correlated with the fact that Green Mussel is the only species farmed in Jakarta Bay for over three decades, while other species such as Blood Cockle and Oriental Hard Clam are wild- captured shellfish. Thus, Green Mussel becomes the predominant shellfish species in the "traditional" markets and that are likely to be more exposed to viral contamination than other species. In addition, both NoV purified from the positive samples were identified as GII.4. This genotype (GII.4) has been reported worldwide as the predominant strain in the NoV genogroup II (GII) that was responsible for many human NoV-gastroenteritis outbreaks caused by either person-to-person transmission or food contamination (Baert *et al.*, 2009; Bernard *et al.*, 2014; Bull *et al.*, 2006; Fitzgerald *et al.*, 2014; White, 2014; Zheng *et al.*, 2010).

Based on the Decree of the Indonesian Minister of MAF no. KEP.17/MEN/2004 (MMAF, 2004) regarding the Indonesian shellfish sanitation system, shellfish farming activities are prohibited in particular areas which have a high level of faecal contamination in the water (> 300 MPN coliforms/100 ml) and an excessive level of PSP toxin in the shellfish (> 80µg/100 g of shellfish meat). Such activities are also prohibited in areas that have not been assessed for the sanitation compliance. Jakarta Bay plays important roles in different sectors, including the economic, transportation, tourism and fishery sectors, however the Bay has been experiencing heavy pollution from domestic and industrial activities in the surrounding areas (Arifin, 2004; Dsikowitzky *et al.*, 2016; Siregar *et al.*, 2016). Therefore, the Indonesian MMAF has proposed Banten Bay as one of the potential replacements for shellfish growing sites in Jakarta Bay (Andriyanto, 2018). Results from this study which showed that NoV was not found in shellfish from Panimbang market (harvested from Banten Bay which is considered a less polluted area), support this strategy.

Enteric viruses including NoV were not considered as potential microbial contaminants of raw or frozen shellfish in the Indonesian shellfish sanitation system (MMAF, 2004) as well as in the standard processing of frozen (SNI 3460.1 to 3: 2009) and canned shellfish (SNI 3919.1-3:2009)(BSN, 2009). Moreover, regulations that contain minimum safety requirements specifically for viral contamination parameter in fresh shellfish sold in Indonesia do not exist. Therefore, the quantitative microbial food safety risk assessment (QMFSRA) to estimate the risk of NoV infection from consuming shellfish from Indonesia, presented in Chapter 5, could provide science-based information to assist Indonesian regulatory bodies to establish relevant regulations and develop a management control system for NoV in shellfish.

In this study, a risk assessment of NoV in shellfish from Indonesian markets was performed to estimate risks and to provide mitigation strategies. Since several data such as information on the Indonesian NoV outbreak cases, incidences of NoV illness related to shellfish consumption in Indonesia, and the proportion of shellfish species consumed by the Indonesian population, were not available, a deterministic approach was used to develop the risk assessment. To estimate the risk of

NoV cases per year in Indonesia due to consumption of contaminated shellfish, some data from previous studies and adjusted parameters (based on several assumptions and worst-case scenario) were incorporated, while the data for NoV inactivation by thermal inactivation (Chapter 3 of this thesis) was used to calculate the potential NoV reduction after handling and cooking of the commodities.

The potential NoV-contaminated servings of shellfish per year in Indonesia were estimated as 8.17 million servings. The expected number of infections per year due to the consumption of NoV-contaminated shellfish without pre-cooking step in Indonesia was estimated to be 100-folds higher than the pre-cooked shellfish. This risk estimate was based on the assumed proportion of shellfish cooking methods and the worst-case scenario *i.e.*, the highest NoV contamination level, the lowest average of extraction recovery and the highest prevalence data, were used as the input parameters. By the non-pre-cooked following with mixed cooking method assumption and the worst-case scenario, the estimated NoV attack rate (100 cases per 100.000 population) per year in this study were higher than the estimated HAV attack rate in Spain (Pintó *et al.*, 2009). The estimated attack rate of HAV in Spain was calculated using an assumption of mixed format of shellfish consumption (*i.e.*, uncooked, lightly and well-cooked). However, when the pre-cooking was included in the assumption in the risk calculation as an additional step before the different cooking method, the estimate attack rates of NoV due to shellfish consumption in Indonesia was comparable to the estimate of HAV attack rates in Spain (Pintó *et al.*, 2009). Moreover, the estimated NoV attack rates in the current study was lower than those of estimated NoV incidences in UK due to consumption of contaminated raw shellfish (ICMSF, 2018) or the recorded enteric outbreak due to shellfish consumption in China (Halliday et al., 1991). It is worth noting that when the pre-cooking is applied before cooking step (with different methods *i.e.,* boiling, steaming, stir-frying or “mixed”) by consumer or frozen shellfish producer in Indonesia, this step could potentially reduce the incidences of NoV outbreak due to shellfish consumption, and prevent the enteric viruses outbreak such as the reported case in China (Halliday *et al.*, 1991).

6.2. Conclusion

This thesis reported the application of RT-qPCR with enzymatic pre-treatment (RNase followed by RNasin) as a reliable method to quantify infectious viral particles (NoV and MS2) for inactivation studies in both buffered media and mussel matrix. The proposed method was also able to assist the NoV quantification in the prevalence study, which were used to support the QMFSRA of NoV in shellfish sold in fish markets in Indonesia. In general, MS2 has different resistance than NoV toward heat and ClO_2 treatment, thus this bacteriophage may not be the best candidate as a NoV surrogate especially for inactivation studies. Results from the viral inactivation studies confirmed the presence of a matrix effect and tailing phenomenon during the treatment. Hence, the non-linear model such as Biphasic model is suggested as a robust model to be applied to predict and to calculate the thermal inactivation kinetics, while Hom's model is considered as the best model to predict ClO_2 inactivation kinetics of the virus. The improved quantification method (RTqPCR with enzymatic pre-treatment) could be used to minimise over or underestimation of NoV risk in shellfish, while *in-vitro* assay has not been available as to quantify the infectious NoV. Understanding the kinetic of the viruses could also support the evaluation of proposed control measures to reduce or to eliminate NoV contamination. Further incorporation of these information into QMFSRA could finally contribute to a better estimation of the risk NoV illnesses in a given population.

The prevalence study indicated the presence of NoV GII.4 in Green Mussel (*Perna viridis*) harvested from Jakarta Bay. This genotype is also the most common cause of NoV infection worldwide, and this highlights the importance of regular monitoring and surveillance of NoV in shellfish products (before they are distributed) in addition to the well-established monitoring of biotoxin, heavy metals and coliforms in these shellfish growing sites.

The risk assessment suggested that the application of heat treatment (boiling the pre-marketed shellfish) can be used as a control measure to reduce the number of contaminated NoV, and thus lower the risk of NoV infection. Besides, based on the evaluation of ClO_2 efficacy to reduce viral

contamination, this substance could potentially be used as a disinfectant during shellfish handling and processing to reduce NoV contamination from the secondary route (from infected food handler and cross-contamination). However, further studies which incorporate results from the ClO_2 inactivation study into the shellfish processing plan in Indonesia is needed to estimate the risk reductions after application of this treatment.

To overcome the limitations from the current QMFSRA, scientific investigations on the NoV dose-response relationship in Indonesia is needed. Furthermore, integrated approaches to collect and to record information on the proportion of shellfish consumption format of the Indonesian consumers could enhance the accuracy and validity of the NoV risk estimate. Despite the limitations in the risk assessment of this study, this thesis provided science-based evidence which can be applied to improve the management of the quality and safety of shellfish from food-borne NoV, in Indonesia and especially from “traditional” markets in Jakarta.

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